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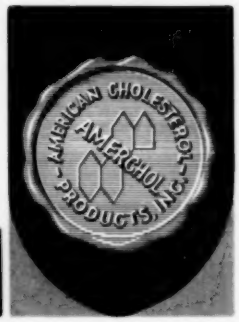

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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The Chemistry of *Ambrosia maritima* L.

I. The Isolation and Preliminary Characterization of Ambrosin and Damsin*

By HAMED ABU-SHADY† and TAITO O. SOINE‡

***Ambrosia maritima* L., used as a popular drug in Egypt, was studied to determine whether it contained active therapeutic principles. Two crystalline compounds, named ambrosin and damsin, were isolated. They were shown to contain a carbonyl group and a lactone group, but upon pharmaceutical examination were found to exhibit low activity for the tests made.**

FOR MANY years, *Ambrosia maritima* L., fam. Compositae (Arabic, Damssissa) has enjoyed some reputation as a popular drug in Egypt. As a common weed, it has been used by the populace in the form of a decoction as a stimulant, stomachic, and cordial drug. Among other miscellaneous uses it has also been described as checking nasal bleeding, alleviating flatulence and controlling blood in the sputum. These uses are undoubtedly based on a folklore background and have no scientific verification. Recent researches, however, have revealed a number of therapeutically active drugs among the herbs growing wild in Egypt. Among these perhaps the most notable are *Ammi visnaga* L. which contains principles useful in angina pectoris (1) and *Ammi majus* L., of which the effectiveness in leukoderma can also be related to crystalline principles (2). Because the therapeutic activity in both of the above cases has been shown to have a relationship to the definite chemical compounds that they contain, a study of *Ambrosia maritima* L. was undertaken to determine whether it, too, might contain active principles.

Ambrosia maritima L. is an annual herbaceous plant widely distributed throughout the Mediterranean region. The plant attains 30–90 cm. in height and is usually very hairy. The dried plant has a characteristically disagreeable odor and is very bitter. Very little is known about the

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The various analyses have been performed by one of the following services: Eli Lilly and Co.; Clark Microanalytical Laboratories (Urbana, Ill.); Microanalytical Laboratory (School of Chemistry, University of Minnesota, Minneapolis).

chemistry and pharmacological activity of *Ambrosia maritima*.[1]

In the present paper, a preliminary chemical and pharmacological study of the constituents of *Ambrosia maritima* L. is described. Two crystalline constituents were isolated and, because these two compounds appear to be the principal constituents of this nature in the plant they have been provisionally named *ambrosin* and *damsin*.

EXPERIMENTAL

Material.—The crude herb, consisting mainly of the flowering tops, was obtained from an "attar" (druggist) in Cairo, Egypt. The material as such was suitable for extraction procedures after being reduced to a no. 20 powder in a Schutz-O'Neil disintegrator. It was noted that, during the grinding, the skin of the operator was considerably irritated and there there was a definite tendency to cause nausea and vomiting as well as excessive sneezing.

Extraction with Alcohol.[2]—One kilogram of the powdered material was refluxed for two hours with 2 L. of alcohol. The alcoholic extract was filtered and the marc again refluxed with the same volume of alcohol for an equivalent period of time. This procedure was repeated four times using fresh alcohol for each extraction. The alcoholic extracts were then combined and concentrated under reduced pressure to a semisolid residue. The soft alcoholic extract thus obtained was digested with a large volume of boiling water and the dark-colored solution filtered while hot. The filtrate was treated with 5-cc. portions of a saturated solution of lead acetate until no further precipitate was obtained. The solution was then boiled and filtered while hot. Excess lead acetate was removed from the solution as lead sulfate by the careful addition of dilute sulfuric acid and subsequent filtration. The lead-free filtrate was transferred to a separatory funnel and shaken several times with small volumes of chloroform until no more extractive was obtained in the chloroform. The chloroform extracts were combined, washed with water, and dried over anhydrous sodium sulfate. The yellowish syrupy residue, left after the removal of chloroform in a current of air, failed to crystallize after being kept in a desiccator for a week under vacuum. The residue was treated with the least volume of boiling water, filtered while hot and the aqueous solution placed in the refrigerator overnight. White crystals (6.5 Gm.) were obtained in this way with m. p. 80–135°.[3] These crystals were recrystallized twice from ether and once from alcohol to give a product melting at 146°. The ethereal mother liquor, on concentration and chilling, yielded a small additional quantity of the same product. Petroleum ether[4] was added to the ethereal mother liquor, after removal of the above crystals, until it was slightly turbid. Refrigeration of the solution resulted in the formation of 2 Gm. of white prisms, m. p. 95–98°. Further addition of petroleum ether together with cooling yielded an additional 1.5 Gm. of the same crystals. Repeated crystallization of these two crops of crystals from a mixture of ether and petroleum ether gave colorless prisms, m. p. 102°.

Extraction with Petroleum Ether.—Ten kilograms of the powdered herb was completely exhausted with petroleum ether in a Lloyd extractor (100 hours, approximately). The petroleum ether extract was decanted from the greenish-colored, resinous, semicrystalline substance (65 Gm.) that had separated. The decanted liquid was concentrated to a small volume and kept in the refrigerator overnight. Another 15 Gm. of a similar semicrystalline material was obtained. The combined solids were digested with 9 L. of boiling water and the aqueous solution was filtered while hot.[5] The filtrate, after standing overnight in the refrigerator, yielded 40 Gm. of almost white prisms, m. p. 90–130°. These crystals were recrystallized three times from alcohol to yield 10 Gm. of product, m. p. 146°. These were found to be identical with the crystals obtained previously by alcohol extraction and melting at the same point. A small additional amount of this product could be obtained by concentration and cooling of the combined mother liquors. The green-colored mother liquor was then diluted with an equal volume of water and the milky suspension was kept for forty-eight hours in the refrigerator. The crystalline deposit (24 Gm.) that formed was collected and dissolved in about 2 L. of petroleum ether (b. p. 60–80°). The volume of petroleum ether was reduced to 1 L. and the concentrated solution left to cool in the refrigerator. By this process 20 Gm. of colorless prisms, m. p. 97–101°, were obtained. Recrystallization of the product from alcohol raised the melting point to 102° and they were found to be identical with the similarly melting substance obtained by alcohol extraction.

The extraction procedures showed that the flowering tops of *Ambrosia maritima* L. contained at least two definitely crystalline compounds, viz., the substance m. p. 146°, and the substance m. p. 102°. These principles have not been previously recorded in the literature and the authors suggest the name *ambrosin* for the substance m. p. 146° and the name *damsin* for the substance m. p. 102°.

Anal.—Calcd. for (ambrosin) $C_{15}H_{18}O_5$: C, 73.15; H, 7.37; C-methyl (1) 5.76. Found: C, 73.06; H, 7.33; C-methyl, 5.88. Saponif. Equiv.: Calcd. for $C_{15}H_{18}O_5$: 246.31. Found: 255.30. Mol. Wt. (Rast): Calcd. for $C_{15}H_{18}O_5$: 246.31. Found: 263.3.

[1] I. R. Fahmy and M. Darwish in *Bulletin de la Fédération Internationale Pharmaceutique*, 23e Année No. 3, 1949, have published their account of a detailed botanical and preliminary phytochemical analysis of this plant. In the course of their studies they isolated a crystalline principle, m. p. 82–84°, to which they assigned the formula $C_{15}H_{18}O_5$ on somewhat questionable analytical data and which they named "ambrosin." It is not known whether their compound is the same as our ambrosin, m. p. 146°, with the same formula.

[2] Unless otherwise specified the term "alcohol" indicates ethyl alcohol (95% C_2H_5OH by volume).

[3] All melting points are uncorrected and were determined on a Fisher-Johns melting point apparatus.

[4] Petroleum ether will be taken to indicate the fraction b. p. 30–60° unless otherwise specified.

[5] To eliminate the possibility that any changes take place in the products isolated due to the boiling water, the two crystalline principles were prepared by a lengthy and tedious crystallization from the original semicrystalline residue in the petroleum ether extract.

Anal.—Calcd. for (damsin) $C_{15}H_{20}O_3$: C, 72.50; H, 8.12; C-methyl (1), 6.05. Found: C, 72.65; H, 8.03; C-methyl, 5.93. Saponif. Equiv.: Calcd. for $C_{15}H_{20}O_3$: 248.31. Found: 250. Mol. Wt. (Rast): Calcd. for $C_{15}H_{20}O_3$: 248.31. Found: 250.

The Chemistry of Ambrosin

Ambrosin crystallizes in colorless, odorless prisms. The crystals are extremely bitter and are very sparingly soluble in water. Ambrosin is freely soluble in chloroform and to a lesser extent in alcohol or benzene, but very sparingly soluble in ether and petroleum ether. It is insoluble in cold dilute 2% sodium hydroxide solution but is readily soluble on gentle warming. Acidification of such a solution resulted in the regeneration of ambrosin. On the other hand, after being refluxed with a 10% solution of sodium hydroxide for thirty minutes it was impossible to regenerate ambrosin from the yellow-colored solution, a resinous product being obtained instead. Ambrosin is insoluble in dilute acids even on warming, but is soluble in sulfuric acid with the formation of a yellow color changing to dark brown on warming. It is also freely soluble in glacial acetic acid. On heating, ambrosin chars and leaves no residue on incineration.

Standing in light over a period of time, ambrosin crystals turn yellow in color, but the melting point of the crystals is not affected appreciably. The colorless form could, however, be readily obtained from the yellow crystals by recrystallizing them from alcohol.

Combustion of carefully dried samples of ambrosin has given good evidence for the suggested empirical formula of $C_{15}H_{18}O_3$. It is noteworthy, however, that if the samples were not well dried following crystallization from alcohol, the analytical figures were variable but, nevertheless, pointed to the retention of approximately one mole of alcohol of crystallization. Unfortunately, we were unable to obtain satisfactory analytical data to support the formula $C_{15}H_{18}O_3 \cdot C_2H_5OH$ for the alcohol-crystallized and undried samples of ambrosin. The following analytical data were the best obtainable from our samples.

Anal.—Calcd. for $C_{15}H_{18}O_3 \cdot C_2H_5OH$: C, 69.84; H, 8.27. Found: C, 70.16; H, 7.81.

Qualitative Tests.—Sodium fusion showed the absence of nitrogen, halogen, and sulfur. Negative tests were obtained on the addition of the following reagents to a saturated aqueous solution of ambrosin: ferric chloride T. S., sodium hypoiodite (iodoform test), Mayer's reagent (*cf.* damsin). Positive tests were obtained with bromine water T. S., potassium permanganate T. S., Tollen's solution and 2,4-dinitrophenylhydrazine T. S.

A 5% bromine solution in carbon tetrachloride was decolorized when added dropwise to a cold 5% solution of ambrosin in the same solvent. No fumes were noted until the solution was warmed.

Specific Rotation.—The specific rotation of ambrosin was determined in alcohol with a Schmidt-Haensch polarimeter and found to be $[\alpha]_D^{22} = -154.50°$. The concentration of the solution was 0.02 Gm. of ambrosin per cc. of alcoholic solution.

Reduction.—Ambrosin (2 Gm.) was dissolved in 80 cc. of methyl alcohol and placed in a conventional Parr catalytic hydrogenation apparatus with 0.25 Gm. of a 10% palladium-on-charcoal catalyst under an initial pressure of 41 lb. of hydrogen. Reduction took place within approximately fifteen minutes but the reduction was permitted to proceed for approximately one hour. The solution was filtered from the catalyst and the filtrate then concentrated and cooled. Crystals (0.5 Gm.), m. p. 150–160°, separated immediately and were removed by filtration. Crystallization from about 50 cc. of ether yielded a product, m. p. 164–165°. That these crystals could not have been the original ambrosin was shown by a depression of melting point when a mixed melting point was carried out. Four recrystallizations from ether yielded an analytical sample, m. p. 165°. From the analytical data it is proposed that this compound be named *dihydroambrosin*.

Anal.—Calcd. for $C_{15}H_{20}O_3$: C, 72.50; H, 8.12; C-methyl (2), 12.10. Found: C, 72.62; H, 8.17; C-methyl, 12.89.

The mother liquor obtained from the original separation of dihydroambrosin was evaporated almost to dryness to yield 1.2 Gm. of prisms, m. p. below 110°. Recrystallization of these prisms from ether yielded a small additional quantity of dihydroambrosin as well as 0.65 Gm. of prisms, m. p. 105–111°, when petroleum ether was added to the ethereal solution. On repeated crystallization from a mixture of ether and petroleum ether an analytical sample was obtained, m. p. 128°. From a consideration of the analytical data, this compound has been named *tetrahydroambrosin*.

Anal.—Calcd. for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86; C-methyl (2), 12.01. Found: C, 71.91; H, 8.55; C-methyl, 12.55.

Dihydroambrosin was converted to the oxime in the usual manner to give colorless prisms, m. p. 216° (decomp.). It was also converted to the 2,4-dinitrophenylhydrazone to give orange prisms, m. p. 228–229°.

Anal.—Calcd. for (the oxime) $C_{15}H_{21}O_3N$: C, 68.41; H, 8.04; N, 5.32. Found: C, 68.71; H, 8.20; N, 5.39.

Anal.—Calcd. for (the 2,4-dinitrophenylhydrazone) $C_{21}H_{24}O_6N_4$: C, 58.87; H, 5.65; N, 13.08. Found: C, 58.90; H, 5.93; N, 13.12.

Tetrahydroambrosin was converted to the oxime in the usual manner to give colorless, prismatic needles, m. p. 205°, with darkening at 200°. An attempt to prepare the 2,4-dinitrophenylhydrazone resulted in the formation of orange-red prisms, m. p. 172°, which gave analytical values indicating that two moles of the reagent had reacted and split out one mole of water. Only one mole would normally be expected to react.

Anal.—Calcd. for (the oxime) $C_{15}H_{23}O_3N$: C, 67.89; H, 8.73; N, 5.28. Found: C, 67.95; H, 8.72; N, 5.18.

Anal.—Calcd. for (the 2,4-dinitrophenylhydrazone ?) $C_{21}H_{26}O_6N_4$: C, 58.60; H, 6.08; N, 13.00. Calcd. for $C_{27}H_{32}O_{10}N_8$: C, 51.59; H, 5.13; N, 17.83. Found: C, 52.12; H, 4.50; N, 17.63.

Monopiperonylidene Derivative.—Ambrosin (0.5 Gm.), together with 0.5 Gm. of piperonal, was dissolved in 10 cc. of absolute alcohol. Five cc. of a saturated alcoholic solution of hydrogen chloride was then added and the solution was kept at room temperature for forty-eight hours. During this time the yellow-colored solution gradually turned deep red. The solution was then freed of solvent by heating on a steam bath in a current of air. The

dark oil that remained was dissolved in 20 cc. of alcohol with the aid of heat and filtered. Upon standing at room temperature, the solution deposited crystals at which time it was placed in the refrigerator. The crystals obtained (0.4 Gm.) were collected on a filter and found to melt at 220–230°. These crystals were crystallized once from alcohol and twice from glacial acetic acid to give yellow prisms, m. p. 243–244°. They gave a dark, cherry-red color reaction with commercial sulfuric acid, although the pure reagent grade concentrated sulfuric acid failed to give this color reaction even on the addition of a few drops of ferric chloride solution.

Anal.—Calcd. for $C_{23}H_{22}O_5$: C, 73.26; H, 5.86. Found: C, 72.90; H, 6.21.

Bromination.—Ambrosin (1 Gm.) was dissolved in 40 cc. of cooled ethyl acetate and to this solution was added dropwise a solution of bromine in ethyl acetate until a permanent reddish-brown color was imparted. Approximately 600 mg. of bromine was taken up (650 mg. is the theoretical amount required for one double bond). The ethyl acetate was then removed in a current of air and the residue was dissolved in a little alcohol. The alcoholic solution was treated with water until it was slightly turbid and it was then left for three days at room temperature. At the end of this time 0.35 Gm. of colorless crystals had separated and it was noted that the mother liquor was quite acid. These crystals were recrystallized from alcohol several times to yield colorless prisms, m. p. 143–145°. The ultraviolet spectrum of an alcoholic solution of this compound was determined on a Beckmann Model DU quartz spectrophotometer and is reproduced in Fig. 1. On the basis of the analytical data this compound has been named *bromoambrosin*.

Anal.—Calcd. for $C_{15}H_{17}O_3Br$: C, 55.39; H, 5.27. Found: C, 55.38; H, 5.44.

Action of Sulfuric Acid.—Ambrosin (2 Gm.) was mixed with 22.5 cc. of water and 15 cc. of sulfuric acid and then kept at a temperature of 70° for 19 hours. At the end of this time the solution had acquired a greenish fluorescence and a brown color. It was then diluted with 100 cc. of water, transferred to a separatory funnel, and extracted repeatedly with ether. The ethereal extracts were combined, dried, and concentrated to a small volume. Yellowish-colored prisms (0.8 Gm.) separated on standing and were found to have a melting point of 155–158°. Several crystallizations from a mixture of ether and petroleum ether yielded slightly yellow prisms, m. p. 162–163°. The analytical results indicate that the compound is an isomeric ambrosin and, accordingly, it has been named *isoambrosin*.

Anal.—Calcd. for $C_{15}H_{16}O_5$: C, 73.14; H, 7.37. Found: C, 73.21; H, 7.55.

The Chemistry of Damsin

Damsin crystallizes as colorless, odorless prisms which are very bitter and only slightly more water-soluble than ambrosin. Damsin is freely soluble in chloroform, alcohol, and ether, but only slightly soluble in petroleum ether. It is insoluble in cold dilute alkali but is soluble on gentle warming and may be recovered unchanged by acidification of the alkaline solution. As was the case with ambrosin, the application of excessive heat to alkaline solutions of damsin causes changes which give only resins on acidification. It is insoluble in dilute acids but is soluble in strong mineral acids and in glacial acetic acid.

Qualitative Tests.—A sodium fusion of damsin indicates the absence of nitrogen, sulfur, and halogens and incineration of a small sample leaves no residue indicating the absence of metals. Negative qualitative tests were obtained on the addition of the following reagents to a saturated aqueous solution of damsin: ferric chloride T. S., Tollen's solution, sodium hypoiodite (iodoform test). Positive tests were obtained with bromine water T. S., potassium permanganate T. S., Mayer's reagent, and 2,4-dinitrophenylhydrazine T. S. In the case of damsin, as with ambrosin, the addition of a 5% bromine solution in carbon tetrachloride to a cooled 5% solution of damsin in carbon tetrachloride was accompanied by decolorization and without the evolution of hydrogen bromide fumes.

Specific Rotation.—The specific rotation of damsin was determined in alcohol with a Schmidt-Haensch polarimeter and was found to be $[\alpha]_D^{23} = -29.1°$. The concentration was 0.02 Gm. of damsin in each cubic centimeter of solution.

Derivatives.—Damsin (0.25 Gm.) was heated to boiling with a solution containing 0.5 Gm. of 2,4-dinitrophenylhydrazine in 2 cc. of hydrochloric acid and 15 cc. of alcohol. The solution was then allowed to cool to room temperature and crystallization was induced by scratching the inner side of the flask. The yellow crystalline hydrazone was collected and recrystallized several times from alcohol to give an analytical sample, m. p. 225–226°. A mixed melting point determination with the 2,4-dinitrophenylhydrazone of dihydroambrosin did not show a depression of the melting point. However, comparison of the X-ray diffraction patterns[6] of the two derivatives, both crystallized from alcohol, showed them to be dissimilar.

Anal.—Calcd. for $C_{21}H_{24}O_8N_4$: C, 58.9; H, 5.6; N, 13.1. Found: C, 59.2; H, 5.5; N, 12.9.

Attempts to prepare an oxime, semicarbazone, and *p*-nitrophenylhydrazone from damsin in the usual manner were unsuccessful.

Reduction.—Damsin was hydrogenated in essentially the same way as was ambrosin except that an ethereal solution was used. The same products were isolated from this reduction as from the reduction of ambrosin. The identity of the products from the two reductions was established by means of mixed melting point determinations. It was also demonstrated that dihydroambrosin, an isomer of damsin, was more stable to alkali than the latter. This was shown by refluxing it for fifteen minutes with a 2% solution of sodium hydroxide and then obtaining it unchanged by acidifying the alkaline solution.

Piperonylidene Derivative.—Attempts have been made to prepare the piperonylidene derivative of damsin under both alkaline and acid conditions, but it has not yet been possible to prepare a suitable crystalline derivative.

Bromination.—A solution of bromine in ether was added dropwise to a solution of 1 Gm. of damsin in dry ether until a permanent brown color was imparted to the solution. The ether was then

[6] These were kindly determined by W. N. Lipscomb, Associate Professor of Physical Chemistry, School of Chemistry, University of Minnesota.

evaporated from the solution, together with the excess of bromine, in a current of air. The yellow syrup that remained was dissolved in alcohol and the solution was diluted with water until turbid and left to crystallize at room temperature. After a week, crystals formed and were filtered from the solution and recrystallized three times from alcohol to give colorless prisms, m. p. 201°.

Anal.—Calcd. for $C_{15}H_{19}O_3Br_3$: C, 36.98; H, 3.93; Br, 49.2. Found: C, 33.09; H, 3.66; Br, 55.54. Calcd. for $C_{15}H_{17}O_3Br_3$: C, 33.86; H, 3.71; Br, 52.00.

Action of Sulfuric Acid.—When damsin was treated in essentially the same way as was ambrosin (q. v.) a yellow oil was obtained. Although it deposited a few crystals after many efforts to crystallize it, the amount of crystalline material was too small to be utilized.

Ultraviolet Spectra.—The ultraviolet spectra of the compounds in this paper were determined on a Beckman Model DU quartz spectrophotometer in 95% ethanol; $\epsilon = 1/c \log_{10} I_0/I$ where c represents moles per liter. See Fig. 1.

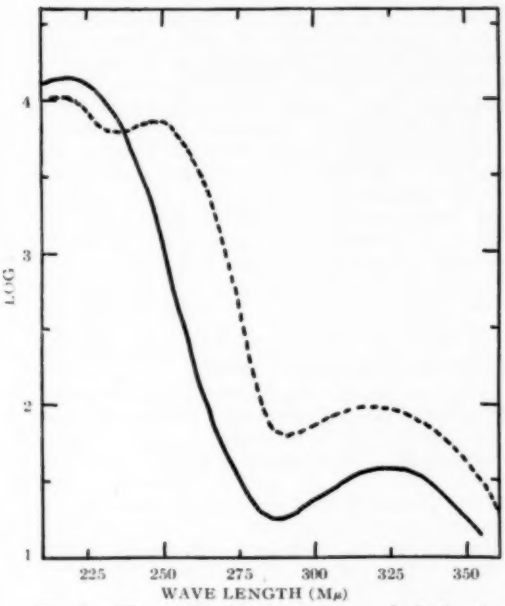


Fig. 1.—Ultraviolet Spectra. (———), Ambrosin; (- - - -), Bromoambrosin.

Infrared Spectra.—The infrared spectra of the compounds in this paper were determined on a Beckman IR2T spectrophotometer with a rock salt monochromator, chloroform as a solvent, and concentrations varying from 3 to 5.7%. See Fig. 2.

DISCUSSION

Ambrosin.—Because ambrosin gave no reaction with ferric chloride solution, it was assumed to contain no free phenolic or enolic hydroxyl groups. The infrared spectrum gave no evidence for phenyl groups or for free hydroxyl groups. Because of the positive tests to bromine and potassium permanganate, and because of the presence of certain bands in the infrared spectrum which are discussed later in this paper, the presence of centers of unsaturation was suspected.

The presence of a lactone group in the ambrosin molecule was suggested by the solubility of ambrosin in alkali on gentle warming followed by its regeneration on acidification. On saponification the compound consumed 1 mole of alkali, although attempts to obtain the free acid by careful acidification met with failure because the original ambrosin was the only product obtained. Likewise, the attempt to prepare an amide failed. However, the infrared spectrum of ambrosin as well as of its reduction products supports the concept of a lactone structure. A carbonyl frequency at 1770 cm.$^{-1}$ is regarded as being typical for a γ-lactone. A very weak band at 3,550 cm.$^{-1}$ is indicative of a very small amount of hydroxyl and is approximately the same relative intensity as the hydroxyl band found in the spectrum of butyrolactone. A similar weak hydroxyl absorption was found at 3,623 cm.$^{-1}$ in a sample of tetrahydrosantonin (known to contain a γ-lactone group) that was run for comparison purposes. It might be well to state at this point that the spectrum of tetrahydrosantonin was held to have very few points of similarity with that of ambrosin, damsin, or their reduction products.

Reduction of ambrosin, which analyzes for one C-methyl group, yields two well crystallized products, namely dihydroambrosin, $C_{15}H_{20}O_3$, and tetrahydroambrosin, $C_{15}H_{22}O_3$. Each of these reduced compounds has double the C-methyl content of ambrosin and both appear to have lost the conjugate unsaturation according to the infrared spectra. An examination of the infrared spectra of both compounds showed the disappearance of the 1,594 cm.$^{-1}$ frequency of ambrosin together with a shift of the carbonyl peak to 1,757 cm.$^{-1}$ in dihydroambrosin and to 1,764 cm.$^{-1}$ in tetrahydroambrosin. The frequency for what is interpreted to be the unconjugated double bond of ambrosin is at 1,675 cm.$^{-1}$ and is found to be intensified in dihydroambrosin at 1,672 cm.$^{-1}$ whereas it has disappeared completely in tetrahydroambrosin. As a matter of interest, the same band is found in the spectrum of damsin at 1,677 cm.$^{-1}$. The analytical and spectral data suggest that a double bond still remains in dihydroambrosin whereas the same double bond has apparently been saturated in tetrahydroambrosin. Although the frequency for the double bond is lower than usual, it is believed to be the only band that would account for an unconjugated double bond and is supported by the presence of an apparently similar band in the spectrum of helenalin (9). More detailed reduction studies have been carried out and will be presented in a subsequent paper.

Qualitative tests for a carbonyl function were positive. Although no crystalline hydrazone, oxime, or semicarbazone was obtained from the reaction of ambrosin with the corresponding carbonyl reagents, dihydroambrosin readily yielded an orange-colored, crystalline 2,4-dinitrophenylhydrazone and a colorless oxime. Tetrahydroambrosin, on the other hand, reacted with hydroxylamine to give the normal oxime but reacted abnormally with 2,4-dinitrophenylhydrazine to give a product which could not have been the hydrazone. The infrared spectrum, of course, supports the presence of a

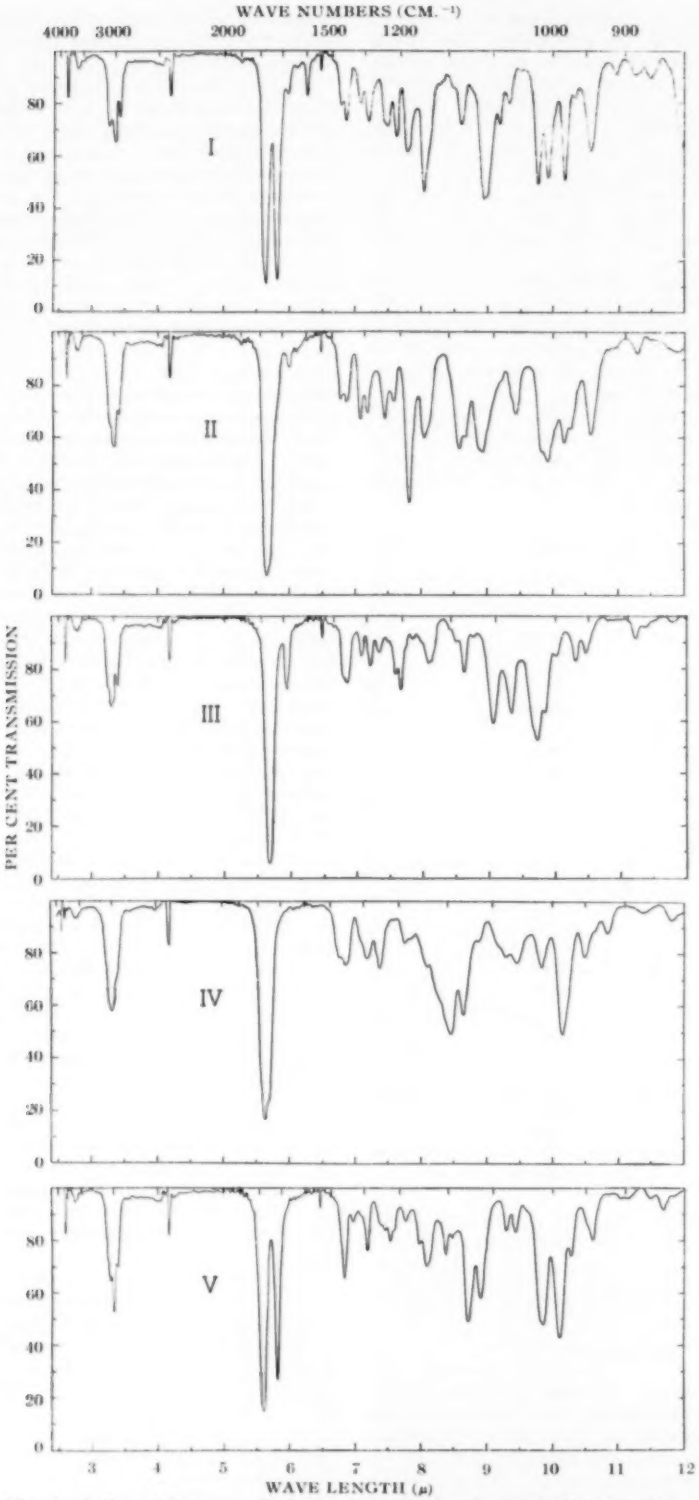


Fig. 2.- Infrared Spectra. I, Ambrosin; II, Damsin; III, Dihydroambrosin; IV, Tetrahydroambrosin; V, Tetrahydrosantonin.

carbonyl group. The presence of a carbonyl group accounts for the third oxygen with the lactone group requiring the other two oxygens.

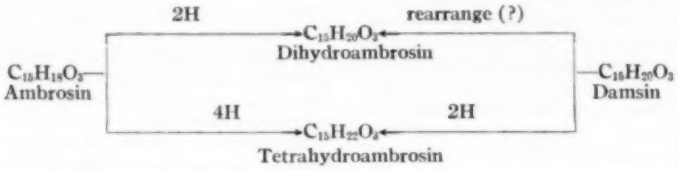
Ambrosin gave no color with Schiff's reagent but moderately reduced Tollen's reagent. This supports the ultraviolet spectrum ($\lambda_{max.1}$ = 217 mμ, $\epsilon_{max.1}$ = 13,465; $\lambda_{max.2}$ = 324 mμ, $\epsilon_{max.2}$ = 36) which is indicative of an α,β-unsaturated ketone. The English workers, Gillam and West (3), have pointed out that isolated carbonyl groups in chromophorically simple molecules usually give rise to an absorption band near 280 mμ having ϵ of the order of <100 whereas in the α,β-unsaturated carbonyl compound this band is displaced to the neighborhood of 300–320 mμ and a new band of much greater intensity appears between 220 and 250 mμ (ϵ = order of 6,000 to 14,000). Literature analyses and work by these workers and Woodward concerning α,β-unsaturated ketones have led to the formulation of certain rules by Woodward (4, 5) that have been valuable in providing structural assignments to many natural products. According to these findings there is a bathochromic shift of *ca.* 10 mμ for each alkyl substituent and one of 5 mμ for an exocyclic double bond. A disubstituted α,β-unsaturated ketone would, by Woodward's rules, have a maximum in the region 235 mμ if no exocyclic double bond were present or $\lambda_{max.}$ = 240 mμ if there were an exocyclic double bond. A monosubstituted α,β-unsaturated ketone has $\lambda_{max.}$ = 225 ± 5 mμ. However, Gillam and West (6) have demonstrated that if the chromophore is a part of a five-membered ring it is correspondingly lower. In the cases of isothujone, dihydrojasmone, and tetrahydropyrethrolone the observed $\lambda_{max.}$ fell short of that predicted by Woodward's rules by 9.5, 10, and 15 mμ, respectively. If the average amount of deviation (−11.5 mμ) is applied as a correction to 235 ± 5, the predicted value for a disubstituted α,β-unsaturated ketone, the corrected $\lambda_{max.}$ for a cyclic α,β-unsaturated five-membered ring ketone becomes 223.5 ± 5 mμ. On this basis, the position of the first maximum in ambrosin suggests that ambrosin is a disubstituted α,β-unsaturated ketone providing that the chromophore is in a five-membered ring.

Bromination of ambrosin in ethyl acetate always gave rise to a monobromo derivative although approximately the equivalent of two bromine atoms was consumed. The acidity of the crystallizing media suggested that possibly the bromine had added across a double bond, but that subsequent splitting out of hydrogen bromide had occurred to regenerate the double bond. Variation of the experimental conditions with a view toward obtaining the intermediate dibromo compound from ambrosin led only to the formation of complex amorphous products. The ultraviolet spectrum of bromoambrosin ($\lambda_{max.1}$ = 248 mμ, $\epsilon_{max.1}$ = 7,025; $\lambda_{max.2}$ = 320 mμ, $\epsilon_{max.2}$ = 95) appears to be characteristic of either an α- or β-bromo-α,β-unsaturated ketone. Woodward, in his original formulation of the rules pertaining to conjugated ketones, tentatively suggested that bromine and alkyl groups would have about the same effect. However, Djerassi and Scholz (7) later commented on the ultraviolet spectrum of Δ^1,2-bromoandrosten-17α-ol-3-one 17-hexahydrobenzoate pointing out that it constituted an exception because the absorption maximum occurred at 255–256.5 mμ rather than at 235 mμ as would have been predicted by the rules. Nussbaum and co-workers (8) have, since then, shown on the basis of their experience with the spectra of aliphatic α,β-unsaturated carbonyl compounds that there is a bathochromic shift of from 21 to 25 mμ (in one case it was 30 mμ) to be expected on the α-bromination of these α,β-unsaturated compounds. The single β-bromo derivative they examined showed a shift of 30 mμ. On the basis of these findings, the 31 mμ shift undergone by ambrosin via bromination could be assigned to the presence of a bromine atom in either the α or β positions. If, indeed, the α-position is occupied by the bromine, as seems very likely, it excludes the possibility of an alkyl substituent α to the carbonyl. The formation of a monopiperonylidene derivative of ambrosin implies the presence of an active methylene group adjacent to the carbonyl and, therefore, excludes the possibility of an alkyl substituent on that carbon.

The infrared spectra of ambrosin and damsin revealed supporting evidence for the presence of a conjugated carbonyl group. The carbonyl peak in ambrosin is at 1,718 cm.$^{-1}$ accompanied by a relatively weak absorption at 1,594 cm.$^{-1}$ which may be related to a conjugated C=C. Inspection of the spectrum of damsin leads to the finding that there is a single carbonyl peak which, judging from its intensity and asymmetry, is composed of two overlapping bands. The peak centers at 1,766 cm.$^{-1}$ (typical for lactones) and the other overlapping group is at 1,754 cm.$^{-1}$ and must, of necessity, be the carbonyl peak. It is also to be noted that the 1,594 cm.$^{-1}$ peak of ambrosin is not present. Inasmuch as both ambrosin and damsin yield identical products on mild reduction it may be assumed that they possess the same carbon skeleton. On this assumption it becomes feasible that damsin is another dihydroambrosin in which the conjugated unsaturation is not present. Adams and Herz (9) observed a strikingly similar change in the infrared spectrum of helenalin on reduction. Helenalin, when reduced to tetrahydrohelenalin, undergoes a change in its carbonyl peak from 1,702 cm.$^{-1}$ to 1,737 cm.$^{-1}$ whereas a weak absorption at 1,580 cm.$^{-1}$ (ascribed to highly conjugated C=C) disappears. They point out that a carbonyl maximum of 1,737 cm.$^{-1}$ (in tetrahydrohelenalin) is unusually high for unconjugated acids, aldehydes, and ketones but that it is characteristic of cyclopentanones. In a similar vein, the frequency 1,702 cm.$^{-1}$ (in helenalin) is high for conjugated carbonyl and the frequency 1,580 cm.$^{-1}$ is unusually low for comparable bands in normal α,β-unsaturated ketones. They cite the work of Jones and coworkers (10) in which a number of conjugated 17-keto steroids had carbonyl maxima at 1,716 cm.$^{-1}$ whereas the corresponding saturated ketones had the maxima shifted to the range 1,735–1,745 cm.$^{-1}$. On this basis, Adams and Herz suggest the possible presence of a cyclopentenone ring in helenalin. Similar conclusions, based on our infrared evidence, may be drawn concerning the presence of such a ring in ambrosin also.

Damsin.—The presence of a carbonyl group, suspected from the results of the qualitative testing,

was confirmed by the formation of a 2,4-dinitrophenylhydrazone from damsin. Whether this derivative is identical with the same derivative of dihydroambrosin is not known at present in view of the conflicting evidence provided by mixed melting point determinations and X-ray diffraction patterns. Although the oxime, semicarbazone, and *p*-nitrophenylhydrazone apparently formed, they were not amenable to crystallization. However, it was found that damsin yielded the same products (dihydroambrosin and tetrahydroambrosin) as ambrosin did when it was subjected to a mild reduction with palladized charcoal as a catalyst. The following scheme shows the relationship of these reduction products to both ambrosin and damsin.



As has been pointed out previously, both dihydroambrosin and tetrahydroambrosin form typical carbonyl derivatives thus confirming the presence of the carbonyl group in damsin. The spectrum of damsin in the infrared suggests the lack of conjugation also.

Damsin, as is the case with ambrosin, dissolves in dilute alkali with warming but is regenerated by acidification. That the lactone group is retained by dihydroambrosin becomes evident from the fact that it dissolves in warm dilute alkali also and is regenerated by acidification. It is to be noted that dihydroambrosin appears to be much more stable to alkali than either ambrosin or the isomeric damsin. The infrared spectrum of damsin shows a single carbonyl band, but the intensity and width of the band suggest two overlapping bands from two carbonyl groups. The frequency at 1,766 $cm.^{-1}$ is again typical for a γ-lactone and the same weak hydroxyl contribution (attributable to the lactone group) is found at 3,550 $cm.^{-1}$ as was found in ambrosin. Damsin consumes 1 mole of alkali on saponification as would be expected from a lactone. Attempts to prepare the amide or free acid from damsin have led only to compounds that would not crystallize or to regeneration of the original damsin. The presence of a double bond and some of its characteristics seems to be well established. Several observations are in accord with the concept of a double bond as a part of a terminal methylene group. For example, although damsin itself analyzes for only one C-methyl group, its products from the hydrogenation, namely, dihydroambrosin and tetrahydroambrosin, have double the C-methyl content of damsin. In this respect the situation is the same as with ambrosin, which has been discussed, and is believed to constitute good evidence for a terminal methylene group in both ambrosin and damsin. A terminal methylene group is also suggested by the infrared analysis (spectral curve not shown) in which an absorption peak at 815 $cm.^{-1}$ is attributed to this group. It was hoped that a dibromide could be made by the simple addition of bromine to damsin, but no products have yet been isolated that could be definitely characterized. Further studies on the reduced products, as mentioned previously, have been carried out and will be reported in a future communication.

Pharmacological Data

The pharmacological data were provided by Mary A. Root of Eli Lilly Research Laboratories, Indianapolis, Ind. The solution of ambrosin used was prepared by dissolving 50 mg. of the compound in 2 cc. of alcohol and diluting it to 50 cc. with 0.9% sodium chloride solution. A concentration of 0.1% was the highest that could be obtained. A solution of damsin with a maximum concentration of 0.4% was prepared by dissolving 200 mg. of the compound in 4 cc. of alcohol and diluting it to 50 cc. with 0.9% sodium chloride solution. The tests performed and the results obtained are recorded in Table I.

Comments.—The combination of low solubility and low activity makes these compounds of little interest pharmacologically. They have not been tested specifically for curare-like activity, but the results obtained with other tests would make such activity appear very unlikely. The activity on the isolated ileum might indicate a parasympathomimetic action but the fact that atropine does not block the blood pressure fall caused by damsin rules out any acetylcholine-like action.

SUMMARY

The isolation of two crystalline principles, provisionally named *ambrosin* and *damsin*, from *Ambrosia maritima* L. has been described. Ambrosin crystallizes as white prisms, m. p. 146°, $[\alpha]_D^{22} = -154.50°$. Damsin crystallizes as white prisms also, m. p. 102°, $[\alpha]_D^{22} = -29.1°$. The formula of ambrosin has been established from the analytical data as $C_{15}H_{18}O_3$ and that of damsin as $C_{15}H_{20}O_3$.

It has been demonstrated that, on reduction, both ambrosin and damsin yield the same products, namely, dihydroambrosin, $C_{15}H_{20}O_3$, m. p. 165°, and tetrahydroambrosin, $C_{15}H_{22}O_3$, m. p. 128°. The presence of a terminal methylene group ($>C = CH_2$) in both ambrosin and damsin is suspected, largely on the C-methyl content of di- and tetrahydroambrosin.

A γ-lactone group is believed to be present in both ambrosin and damsin on the basis of their alkali solubility, saponification equivalent, and infrared spectral data.

A carbonyl group has been shown to be present in both ambrosin and damsin and, together with

TABLE I.—PHARMACOLOGICAL TESTS PERFORMED AND RESULTS OBTAINED

Tests Performed	Results	
	Ambrosin	Damsin
Blood sugar, rabbit	Doses of 5 mg./Kg., i. v., no effect in 2 animals	Doses of 20 mg./Kg., i. v., slight increase (30 mg. %) in 2 animals 2–3 hr. after injection. Increase not maintained
Blood pressure, cat	Doses of 15–20 mg./Kg., i. v., no effect	Doses up to 10 mg./Kg., i. v., no effect; 10–20 mg./Kg., slight fall not blocked by atropine
Respiration, cat	Doses of 15 mg./Kg., i. v., slight, transient slowing occurred	No effect
Heart rate, cat	No effect	No effect
Nictitating membrane,[a] cat	No effect	Doses of 10 mg./Kg., i. v., caused slight relaxation of stimulated membrane which might indicate slight ganglionic blocking
Toxicity (intravenous), mice	Doses of 40 mg./Kg. killed 2 out of 20 animals[b]	Doses of 80 mg./Kg. killed 2 out of 20 animals. Doses of 100 mg./Kg. killed 4 out of 20 animals[b]
Blocking action, cat	None against acetylcholine, epinephrine, histamine	None against acetylcholine, epinephrine, histamine
Uterus isolated: rabbit, guinea pig	No effect	No effect
Ileum isolated: rabbit	Slight stimulation with a concentration of 1:500,000	Slight stimulation with a concentration of 1:100,000
Guinea pig	No effect	No effect

[a] Stimulated indirectly via cervical sympathetic nerve.
[b] The low solubility made it impossible to determine the LD_{50} of these compounds.

the lactone group, satisfactorily accounts for the three oxygens in these compounds. On the basis of infrared and ultraviolet data it appears that ambrosin contains a conjugated carbonyl group whereas damsin does not have such a group. Ambrosin apparently contains the unconjugated double bond which is present as a terminal methylene group in addition to the center of unsaturation in the conjugated system. The conjugated system is further substantiated by the ultraviolet absorption characteristics of bromoambrosin.

A monopiperonylidene derivative, prepared from ambrosin, implies the presence of an active methylene group adjacent to the carbonyl group. It has not been possible to prepare such a derivative from damsin in a crystalline state.

Certain characteristics in the infrared and ultraviolet data point to the possible presence of a cyclopentenone ring in ambrosin, and a cyclopentanone ring in damsin.

The compounds have been examined pharmacologically and have been shown to be of low activity in all of the tests performed.

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The Properties of Iodine in Solutions of Surface-Active Agents*,†

By NASEEM A. ALLAWALA and SIDNEY RIEGELMAN‡

Iodine solubilized in a surface-active agent solution is shown to be an effective antibacterial agent. An equation is given by which it is possible to calculate the equilibrium concentration of iodine freely dispersed in water (in contrast to micellular iodine). Correlation between this calculated concentration of iodine in water and the 99 per cent killing time is presented. Preliminary data further show the solution to be devoid of most of the disadvantages found in the common iodine solutions.

IODINE has often been called an ideal antiseptic (1), but its use has been curtailed by disadvantages which arise from its nature and the way in which it is used (2). The irritation produced upon application and its corrosive and staining properties are some of the complaints most commonly made against the commercial antiseptic preparations containing elemental iodine (2). Iodine which has been solubilized by surface-active agents was found to be devoid of most of these disadvantages yet retains many of its ideal antiseptic properties. For this reason, details on solubilization of iodine and the properties of the solubilized iodine are discussed below.

IODINE PREPARATIONS

The effectiveness of iodine as an antiseptic has been summarized by several workers (2–5); among the reports made, the most recent and comprehensive is by Gershenfeld (5). Unlike most other antibacterial agents in use, iodine is about equally effective against various test organisms (2), and it has been shown to possess good fungicidal and virucidal properties (6, 7). Furthermore, iodine kills immediately rather than by a long period of stasis (8, 9). Because of these remarkable properties, iodine has been recommended for use as an antiseptic for skin wounds, as a sterilizing agent for equipment and apparatus, as a prophylactic and therapeutic agent in various infectious diseases, and as a disinfecting agent for drinking and swimming pool water supplies (5).

Iodine in aqueous or hydroalcoholic solutions has had official status since 1830. In the past, the numerous changes in the formula of the official iodine preparations have been directed toward minimizing the disadvantages mentioned, and it should be pointed out that as late as June, 1952, Goldstein (10) recommended further changes in the iodine content of the official solutions to prevent iodine crystallization at low temperatures and upon dilution. Most of these changes have been directed toward the pharmaceutical problems of formulation, while neglecting the general physical, chemical, and bacteriological properties of iodine. It may, therefore, be profitable to review some of the properties of the official and the recommended iodine solutions.

Saturated solution of iodine in water is an effective antiseptic preparation. However, due to the low solubility of iodine in water (33 mg. in 100 cc. at 25°), the amount reacting with bacteria or extraneous protein matter rapidly depletes the solution, thus seriously limiting the usefulness of such a solution.

Iodine tincture U. S. P. XIV, strong iodine tincture N. F. IX, and other similar preparations increase the amount of iodine in solution by incorporating some miscible organic solvent, such as alcohol. From this standpoint of avoiding the irritation which results when an iodine preparation is applied to open wounds, cuts, or abrasions, the elimination of alcohol has been considered desirable (11). The alcohol produces a rapid rate of evaporation which has been regarded as an advantage by some workers (12, 13). However, the solid iodine residue left behind stains the skin and proves to be irritating and corrosive to the injured adjoining tissues.

Iodine in propylene glycol was proposed by Gershenfeld and Witlin (13), who found it necessary to add at least 0.5% potassium iodide to dissolve more than 1.4% iodine. It might be added that a precipitate results upon dilution with water if no potassium iodide is used (14). In this laboratory, it has been found that members of polyethylene glycol series such as Polyethylene Glycol 400 U. S. P. XIV, can dissolve up to 20% of iodine. However, the resulting solutions are unstable and cannot be diluted with water without leading to a precipitate.

In strong iodine solution U. S. P. XIV and iodine solution N. F. IX, the use of organic solvents has been avoided by taking advantage of the solubilizing action of alkali iodides on iodine. Gershenfeld (13) has reviewed some of the complications of such solutions, such as freezing easily which is a serious disadvantage from the commercial viewpoint. They show poor penetration properties, dry only very slowly, and stain hands as well as the clothes with which they may come in contact. In addition, there is a fundamental fact about this preparation which is often overlooked. The equation $I_2 + I^- = I_3^-$ represents the reaction involved in the solubilization of iodine by iodide, and the equilibrium conditions in an aqueous media are represented by

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the equilibrium constant, $K = 1.4 \times 10^{-3}$. Out of the three species present in such systems, only the I_2 shows significant antibacterial activity (15). Since the concentration of I^- determines the concentration of I_2 (in no case can it exceed 33 mg. per 100 cc. of water), the ratio between the alkali iodide and the iodine is of paramount importance. This has been clearly demonstrated by Wyss (15), and its implications are of direct concern in any iodine-iodide formulation (10). Furthermore, when water evaporates or is absorbed, a precipitate is obtained which consists of crystalline I_2 and KI, since the tri-iodide complex does not exist in the solid state (16). Again this produces the local irritation and corrosion experienced with alcoholic tinctures. In any case, excessive iodine is often applied resulting in sublimation when the surface is not covered or severe blistering when the surface is covered with a bandage (2).

The surface-active agents have repeatedly been shown to increase the solubility of certain water-insoluble compounds (17). However, this phenomenon has not been extensively applied to drug formulations. The surface-active agents increase the solubility of drugs above the water solubility by incorporating the drug in molecular aggregates called micelles. The micelles are formed in the bulk of the solution when a definite concentration of the surface-active agent has been exceeded. Unlike miscible solvents as alcohol, the effect of surface-active agents in increasing the solubility of a drug becomes pronounced in relatively dilute solutions, whereas miscible solvents have little effect on the solubility unless high concentrations are used (18). The anionic, cationic, and nonionic surface-active agents possess this solubilizing action on iodine. Since the intent of the present research was not to formulate a commercially suitable preparation, the nonionics were selected for the investigation, because they show better solubilization for iodine and, unlike anionics and cationics, do not possess any bacteriological activity of their own. Some cationics and anionics reacted readily with iodine. Aerosol OT (di-octyl sulfosuccinate) and cetyl trimethyl ammonium iodide show good solubilization properties for iodine; the latter surface-active agent itself is a highly effective antimicrobial agent (19).

The detailed experiments were carried out using Antarox A-400;[1] however, many other nonionic surface-active agents now commercially available may be as satisfactory. The following other nonionics were also found to solubilize iodine: Triton X-100 (Rohm & Haas Co.), Antarox A-402 (General Aniline & Film Corp.), polyethylene glycol 400 monolaurate (Glyco Products Co.), Tween 20 and Tween 80 (Atlas Powder Co.), Brij. 35 (Atlas Powder Co.) and Ethomid HT-60 (Armour). Antarox A-400 is a polyethylene glycol nonyl phenol derivative of high purity (approx. mol. wt. 600). The data reported below are exact for one batch, and the other samples are known to show up to 10% deviation in the amount of iodine solubilized. The commercial product was used without further purification except that moisture and the low-boiling components were removed by vacuum distillation (0.3 mm. Hg pressure) at 60°. This agent is a light brown liquid completely miscible with water and other laboratory solvents in all proportions. It is miscible with most oils, but it is immiscible with light liquid petrolatum U. S. P. Shelanski (20) reports that this agent is nonsensitizing, and in this laboratory it was found to be devoid of any antibacterial activity. Its 1% solution in water shows a cloud point around 56°, but it gives a clear solution upon cooling (21).

[1] Antara Division, General Aniline and Film Corp., New York, N. Y.

PROCEDURES

Determination of Solubility.—One hundred fifty-cubic centimeters of solution of known Antarox concentration in a 250 cc-Erlenmeyer flask was overlaid with 25 to 50 cc. of light liquid petrolatum saturated with iodine. The flask was fitted with a ground glass (pyrex) stirrer similar in construction to that used by Hutchinson (22) for partition studies. After equilibrium was reached (twenty-four to thirty-six hours of continuous stirring at 75 r. p. m. in a 25° thermostat) two 50 cc.-aliquots of the aqueous Antarox layer were withdrawn and titrated with freshly standardized sodium thiosulfate. Aliquot portions of petrolatum layer were withdrawn and assayed colorimetrically using Bausch & Lomb Monochromatic Colorimeter and 505-mμ filter.

The concentration of iodine in petrolatum layer was divided with 28.8 (o/w partition coefficient) to obtain the concentration of "free" iodine in water. The log of the concentration of the free iodine was plotted against the log of the concentration of titratable iodine in the aqueous layer. By varying the concentration of iodine in petrolatum layer, three or four other such points were obtained. As shown in Fig. 1, a straight line can be drawn through these points which when extrapolated to 33 mg. % of concentration of "free" iodine gives the corresponding value for the saturation solubility.

Determination of 99% Killing Time.[2]—Freshly prepared spores of *Bacillus cereus* (*metiens*) were used, and the method described by Marks, *et al.* (23), was followed with the following exceptions: (*a*) the final test solution was adjusted to contain 10^6 spores per cc. (99% killing time corresponded to 10^4 surviving spores per cc.); and (*b*) stock suspensions of spores were prepared by using distilled water instead of buffer.

Determination of 99% Killing Time for 100% Antarox A-400 Containing 20% Iodine.—A sample (10 cc.) of stock spore suspension containing approximately 100 million spores was centrifuged at 5,000 r. p. m. for forty-five minutes. The centrifuge tube was then drained almost free of water by gently inverting the tube. To this a known amount of the test solution was added and the suspension was vigorously stirred; rest of the procedure was same as with other solutions. Simultaneous controls were run using 100% Antarox A-400 and sterile distilled water. After ten minutes of observation, the test solution gave the same count of the surviving spores as those for the control solutions.

Vaginal Instillations.[3]—An aliquot of 0.5 cc. of the test solution was administered to large (700 to 800

[2] We wish to thank Miss Grace Gardner for her help in bacteriological testings.

[3] The pathological observations were kindly made by Drs. E. Lowenhaupt and H. Chan of the Department of Pathology of the University of California.

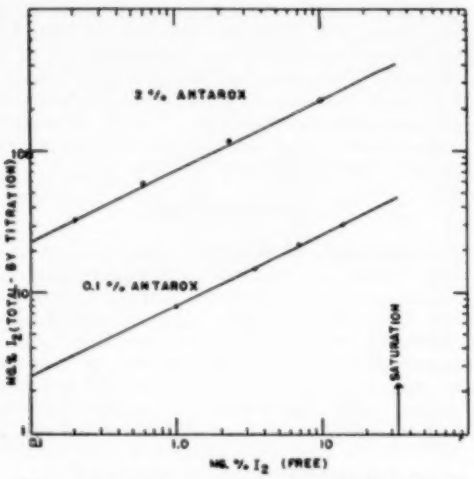


Fig. 1.—A log-log plot showing relationship between free and total (titratable) iodine in solutions of Antarox A-400.

Gm.) guinea pigs by intravaginal instillation. Animals were divided into three groups of four animals each, for every concentration of the iodine solution studied as follows: (*a*) those receiving the vehicle, (*b*) those receiving iodine in Antarox, and (*c*) those receiving I_2 + KI solutions containing a total amount of iodine comparable to that used with group (*b*).

All solutions contained sufficient thickening agent (methylcellulose or pectin) to give proper viscosity so that the solution could be retained in the vaginal cavity. Two animals of each group received only one dose whereas the other two received each at least three doses at one-day intervals. In all cases, animals were sacrificed four days after the last administration.

Skin Irritation Studies.—Hair from the sides of the guinea pigs was clipped and the test solution was applied with rubbing. With one series the painted area was covered, whereas in others, it was left bare. Various concentrations of iodine in Antarox and iodine in potassium iodide and vehicle were used.

Spectrum of Iodine.—Spectral studies were conducted in the spectrographic laboratory, University of California Medical Center, using the Cary Recording Spectrophotometer.

RESULTS AND DISCUSSION

From the solubility studies described above and the partition coefficient studies reported earlier (18), it was possible to develop an equation of the following form to express the solubilization of iodine:

$$X = (m)^a(c)^b \qquad (1)$$

where

X = total amount of titratable iodine in solution (mg. %)
m = concentration of micellular surface-active agent (Gm. %)
c = concentration of free iodine in water (mg. %)
a and b are constants

For Antarox A-400 the constants were experimentally evaluated to be: $a = 0.72$; and $b = 0.57$.

It should be emphasized that this equation is empirical in nature and is only approximate. Nevertheless, it was found to give good agreement between the experimental values for the saturated or unsaturated solutions containing 1 to 50% of Antarox. In all cases, partition experiments were run on all solutions used for bacteriological testing and the values obtained from the partition experiments are those reported in Table I. The correlation between the calculated concentration of iodine in water, c, (with or without added surface-active agent) and the 99% killing time is shown in Fig. 2. This clearly indicates that the 99% killing time is proportional to the equilibrium thermodynamic activity of iodine as represented by the free iodine concentration of water. Table I lists the physical values for various experimentally determined points. Although the total amount of iodine present and the amount of surface-active agent may vary significantly, the free iodine (nonmicellular) determines the biological activity. It may be concluded, therefore, that the bacteriological activity of an antiseptic solution is proportional to the thermodynamic activity of the antiseptic agent, expressed in this case in terms of mg. % "free" iodine, and is not necessarily dependent upon the total amount of that agent.

As Clark (24) points out, an initial lag period is apparent for an antiseptic agent which must penetrate the bacterial wall for its action, when the number of surviving bacteria is followed with the time of application of test solution. With I_2 there

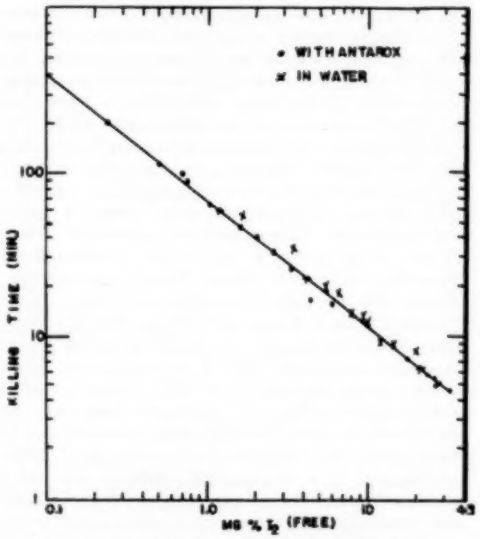


Fig. 2.—A log-log plot showing that 99% killing time [*B. cereus* (*metiens*)] is a function of the concentration of free iodine both in the presence and in the absence of added surface active agent.

TABLE I.—SOLUBILITY, DISTRIBUTION AND KILLING TIME FOR IODINE SOLUTIONS[a]

Conc., W/V % (Ant. A-400)	I_2 in Sol., Mg./100 Cc. Total	I_2 in Sol., Mg./100 Cc. Free	Saturation, Per Cent Total	Saturation, Per Cent Aq.	99% Killing Time Observed	99% Killing Time Corrected
0	28.8	28.8	87	87	5.1	5.1
0	14.8	14.8	45	45	9	9
0	6.7	6.7	20	20	18	18
0	3.5	3.5	10	10	33	33
0	1.2	1.2	3.7	3.7	58	58
0.085	49	33	100	100	3.6	4.8
0.5	165	33	100	100	3.5	4.5
9.3	1200	33	100	100	3.5	4.8
0.085	42	24	89	73	4.4	5.8
1.2	390	26	83	79	4.2	5.5
0.085	6.92	0.7	1.4	2.1	75	103
2	34.4	0.24	0.73	0.73	184	140

[a] All of the determined values are not listed.

is no such lag period and the concentration-time relationship for 99% killing time can be expressed by the following equation which suggests adsorption (24):

$$C^{1/n}T = \text{constant} \qquad (2)$$

where $1/n$ is less than one, T = time in minutes, and C = concentration of iodine in mg./100 cc.

These facts, and the probable mechanism assigned to iodine, make it logical to assume that the rate determining step in the antiseptic action of iodine is its adsorption on the surface of bacteria. In other words, the availability of iodine (18) is expressed by the equation

$$Q = KC^{1/n} \qquad (3)$$

where

Q = availability, i. e., the amount of iodine adsorbed per unit time
K = rate constant
C = instantaneous concentration of iodine in water

Assuming that a constant total amount of iodine, QT, is required to kill 990,000 bacteria, it follows that T, expressed as the 99% killing time, is inversely related to the availability. Under the conditions of this experiment, the equilibrium concentration of iodine in water, c, does not change. The proof of this statement lies in the fact that one million spores used in the above experiment weigh not more than 10^{-6} mg. (25). This weight of protein at saturation will interact with the maximum amount of 10^{-6} to 10^{-4} mg. of iodine (26) whereas concentrations in several milligrams are actually used. Furthermore, the micellular iodine was found to be bacteriologically inactive since 20% iodine in 100% Antarox A-400 showed negligible bactericidal activity. Thus, it appears logical and justified to relate the antibacterial activity to the concentration of I_2 in the water "phase."

This discussion is confirmed from the experimental findings reported here and by Wyss and Strandskov (15) who have used a similar experimental procedure to show that in the iodine-iodide systems, 99% killing time is proportional to the concentration of I_2 in water. The slope of the curve (log 99% killing time plotted against log concentration free I_2) obtained by them is 0.74; whereas in the present experiments, using iodine in water, the slope of 0.75 is obtained. The slope corresponds to $1/n$ in Eq. 2.

With surface-active agents, as in iodine-iodide systems, the solubilized iodine is in equilibrium with the iodine in water and the time required for achievement of this equilibrium is shorter than can be detected in a bacteriological experiment. To test this hypothesis regarding the release of iodine from micelles, standardized sodium sulfite was added, together with the spore suspension in sufficient amount to neutralize instantaneously (27) and completely the aqueous iodine. The 99% killing time observed was exactly the same as it would have been had the same amount of sodium sulfite been added to the test solution before adding the spores. This instantaneous equilibration is essential if the capacity factor (18) is to play a beneficial role.

Unlike iodine-iodide systems, however, the solutions of surface-active agents (less than 50% Antarox A-400) containing iodine can be diluted to any concentration without causing precipitation. Furthermore, iodine from Antarox solutions applied to an open surface does not sublime as readily as with other preparations. This is of considerable practical importance since Nyiri and Janniti (28) have shown that the major portion of the iodine applied to an open surface is lost through sublimation. It was found that under identical conditions where strong iodine solution U. S. P. lost 90% of its iodine through sublimation, a solution of Antarox containing the same initial amount of iodine lost only 40%.

Similarly, it could be shown that the solubilized iodine lacks the irritating properties so commonly encountered with other preparations. A standard adhesive bandage painted with 1/4 cc. of 5% iodine in 50% Antarox was applied to the intact skin on the underside of the forearm. It was left on for as long as six hours without causing any irritation. On the other arm, the official U. S. P. solution was similarly used; it could, however, not be left on for more than one-half hour, due to the unbearable irritation and the severe burn which it caused The surface microscopic examination of the area to which the iodine-Antarox system had been applied gave evidence of the penetration of the solution into the hair follicles. The iodine-iodide system showed only general staining and no comparative ability to penetrate the hair follicles. This preliminary observation on two human subjects was followed by studies on guinea pigs. A portion of the results obtained are reported below, but the detailed find-

ings with other animal work will be reported elsewhere (29).

The guinea pigs receiving more than one vaginal instillation of 1% iodine in 6% Antarox A-400 showed burns and severe vaginitis. This amount of iodine could be administered in 10% (or more) Antarox without any noticeable effects. The animals receiving 0.2% iodine in 0.5% Antarox A-400 were as normal. None of the solutions caused any changes in the skin sections. Iodine-potassium iodide solution caused significantly more irritation than the iodine solution of the comparable concentration in the Antarox. No changes in skin sections were observed with iodine-iodide systems except when the area was covered.

The preliminary observations upon the potentialities of the solubilized iodine in presurgical scrub procedure were conducted by Dr. Cooper Davis of the Experimental Surgery Department of the University of California Hospital, according to the method reported by Price (30). The results using several concentrations of iodine in surface-active agent solutions indicated that such preparations possess an ability to reduce the surface bacterial count to negligible values. However, the bacterial count once again rose to high numbers during a four-hour period of enclosure of the hands in sterile rubber gloves, indicating the lack of any residual activity analogous to that observed with certain preparations of hexachlorophene (G-11). Due to the presence of the surface-active agent, however, the preparations showed satisfactory cleansing properties and did not produce any stains during use.

An additional advantage of the systems containing surface-active agents such as Antarox A-400 and iodine is related to their miscibility with lipoid materials. Pure Antarox is miscible with all vegetable and animal fats and all oils tested. Whereas the tri-iodide complex does not exist in an organic solvent, the iodine dissolved in Antarox A-400 is capable of dissolving in human sebum, which is evident from the skin irritation studies reported above. Further physical evidence to this effect is indicated by the fact that solvents like carbon tetrachloride could not be used for the partition coefficient studies. If such solvents are used, they tend to extract Antarox along with iodine and show an abnormally high "partition coefficient."

There is no question that iodine in surface-active agents is an effective antiseptic agent which has eliminated many of the disadvantages of the former preparations. Nevertheless, certain minor disadvantages with respect to its pharmaceutical application exist for iodine dissolved in Antarox solutions. The solutions are slow drying and leave an oily film of nonvolatile Antarox. By substitution of one of the semisolid surface-active agents or by addition of substances such as methocellulose or polyethylene glycol ointment (31), it is possible to make a satisfactory preparation which can be inuncted into the skin.

Antarox A-400 was selected for the present studies only for the experimental simplifications involved in its use. A frequent complication observed is the fact that under certain conditions (expecially in the presence of excess solid iodine) the solutions lead to precipitate of colloidal iodine. It was noticed that 30 to 40% of the amount of iodine added as solid iodine could not be titrated with sodium thiosulfate after all of the iodine had gone into solution. Since our initial solubilization studies (18) were conducted using a colorimetric method for the assay of iodine, the results obtained are in error as no correction was made for iodine which had reacted with the Antarox molecule.

As shown in Fig. 3, the comparison between the visible spectra of iodine in water, heptane, polyethylene glycol 600, and Antarox indicates that this compound is solubilized by association with the polyethylene glycol portion of the Antarox molecule. The ultraviolet spectrum is also in agreement with the above conclusion and the absorption maxima observed are in agreement with those reported by Osol (32). Besides the acid-base association with polyethylene glycol (32), iodine reacts chemically in such a manner that solubilization properties for iodine are partially destroyed.

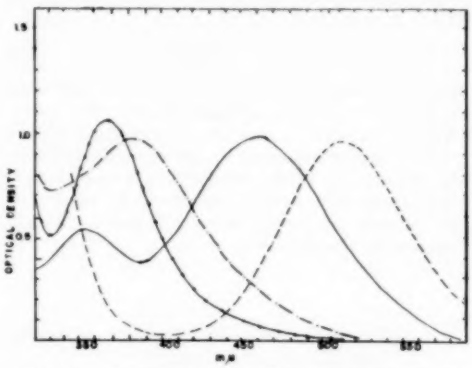
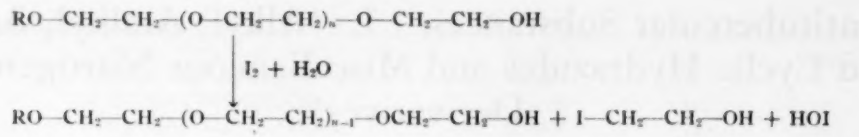


Fig. 3.—Shows the effect of environment on the visible spectrum of iodine. —— water; - - - - heptane; — · — · Antarox A-400; —•—•—•— Polyethylene Glycol 600.

Any reaction postulated must take into account these facts: (*a*) the amount of iodine which so reacts depends upon the number of ethylene oxide groups in the polyethylene glycols, as follows: In all cases 1 cc. of alcohol containing 3.77×10^{-4} moles of iodine was added to 50 cc. of 2% (w/v) solution of polyethylene glycol. At the end of twenty-four hours (a longer period did not increase the amount taken up), the solutions were titrated with standardized sodium thiosulfate solution.

	Non-titratable I_2
P. E. G. 200	4.6×10^{-5}
P. E. G. 600	7.7×10^{-5}
P. E. G. 900	1.08×10^{-4}

(*b*) The reaction involved did not liberate any dissociating acid since no significant change in *p*H could be detected. (*c*) The reaction was not stopped by methoxylation of the terminal hydroxy group in Antarox or polyethylene glycol, indicating that it does not involve oxidation of this group to an aldehyde or acid. (*d*) Upon titration with silver nitrate, half of the iodine which could not be titrated with sodium thiosulfate was found to be organically bound. Therefore, the only possible acretion seems to be:



After neutralization of I_2 with sodium sulfite, ethylene iodohydrin can be characterized in the reaction mixture by conversion to acetaldehyde with lead hydroxide and making a 2,4-dinitrophenyl hydrazone. In the case of Antarox, the ethereal linkage next to the benzene ring seems to be particularly susceptible to cleavage. The resulting phenol would be expected to be easily iodinated.

Since in the absence of excess solid iodine, the reaction seemed to stop with only a small fraction of added iodine already reacted (23%), it was postulated that the addition of HOI may prevent this reaction from starting. Since a solution of HOI free from I_2 can be prepared by dissolving iodine crystals in water and shaking with excess HgO thus pulling the following hydrolysis equilibrium completely to the right

$$I_2 + H_2O = HOI + I^{--} + H^+ \quad K = 10^{-12}$$

The same results can be achieved by adding sodium bicarbonate which does not react with I_2 directly. In this case, some I^{--} is formed which also solubilizes I_2. A solution of HOI so prepared can be used instead of water for dissolving Antarox. Another possibility is to make HOI *in situ*, i. e., by addition of a very small known amount of colloidal HgO to Antarox solution so that when I_2 is added, enough concentration of HOI will be formed to prevent reaction of iodine with Antarox.

Extremely high concentrations of HOI will be required if this reaction is to be successfully blocked in the presence of excess solid iodine. It seems best, therefore, to prepare I_2-Antarox solutions either by the partition method or by addition of alcoholic solution of iodine. Up to 5% alcohol does not seem to effect colloidality of Antarox or the antibacterial activity of I_2-Antarox solution.

In Table I, it will be noticed, that the killing time is listed as "observed" or as "corrected." The values plotted in Fig. 2 are those after correction. By using the above method, it was possible to prepare different solutions containing a known amount of HOI alone or HOI and I_2. Through this method, it was found that HOI in the presence of I_2 is one-sixth as effective against *B. meliens* spores as I_2; Wyss and Strandskov (15) report HOI to be one-third to one-sixth as active as I_2. Since all of the solutions of I_2 in Antarox contain a certain amount of HOI, a correction for it can be made, and if the corrected values are plotted they coincide with those found for iodine solutions in water in the absence of any added Antarox.

SUMMARY

This report indicates that the iodine solubilized in the solutions of surface-active agents has unique properties, and these solutions must be investigated further with respect to their applications. Since the number of available surface-active agents is large, the potential applications of the solubilized iodine systems seem wide and varied.

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Antitubercular Substances. I. Alkyl, Aralkyl, Aryl, and Cyclic Hydrazides and Miscellaneous Nitrogenous Derivatives*

By JOHN SHAVEL, Jr., FREDERICK LEONARD, FREEMAN H. McMILLAN, and JOHN A. KING†

Five series of nitrogenous compounds were synthesized for evaluation of their antitubercular action. None of the seven aliphatic hydrazides or the seven aryl-substituted aliphatic hydrazides showed any appreciable tuberculostatic effect. Several of the fourteen aryl hydrazides did show some effect in inhibiting the growth of *Mycobacterium tuberculosis* strains *in vitro* and *in vivo*, but this effect was not of the same order of magnitude as that exhibited by isonicotinic hydrazide. No usefully significant activity was found in any of the nine compounds which can be considered as hydrazides, in which one or both of the nitrogen atoms form part of a heterocyclic ring. The same results were obtained on a group of twelve miscellaneous nitrogenous compounds consisting of three sulfonhydrazides, two sulfonureas, a sulfonthiourea, three hydroxamic acids, two sulfonhydroxamic acids, and a carbomethoxyhydrazine.

UPON THE announcement by Robitzek, *et al.* (1), of the beneficial clinical effectiveness of isonicotinic acid hydrazide in pulmonary tuberculosis, we undertook the preparation of a number of series of hydrazides and related compounds for their evaluation as tuberculostatic agents. The present communication is a description of those series of hydrazides: (*a*) of aliphatic acids; (*b*) of aliphatic acids substituted by aromatic carbocyclic groups; (*c*) of aromatic carbocyclic acids; (*d*) cyclic hydrazides; and (*e*) miscellaneous nitrogenous derivatives. A few of these compounds, as noted in the tables, have also recently been reported by Bernstein, *et al.* (2), by Buu-Hoi, *et al.* (3–6), and by Gardner, *et al.* (7).

In the tables an *E* in the reference column refers to the experimental section of this paper. The indication of activity is based upon evaluation of the compound *in vitro* against *Mycobacterium tuberculosis*, strains $H_{37}R_v$ and D_4M_3 and by the drug-diet technique in mice infected with the virulent bovine D_4M_3 strain; isonicotinic hydrazide, *p*-aminosalicylic acid, and streptomycin were used as standards. A − in the activity column indicates that the infection showed no significant effect due to the compound, and a ± indicates that the compound exhibited a possibly significant inhibition but that this effect was definitely less than that produced by isonicotinic hydrazide; a + or ++ would indicate that the compound was equally or more effective than isonicotinic hydrazide. Further details on the evaluation of any of the substances will be published, when warranted, by other members of this organization.

With full cognizance of the danger inherent in the drawing of conclusions based upon a limited number of data, one is tempted to conclude from Table I and from the results on the five other aliphatic hydrazides reported by Bernstein (2) that no significant antitubercular activity is likely to be associated with purely aliphatic hydrazides.

The seven carbocyclic-substituted aliphatic hydrazides are listed in Table II and from these results, similarly, there is no indication of activity in this type of compounds.

Table III contains the fourteen aryl hydrazides which we prepared, together with the indication of their activity. It may be noteworthy that of those, no compound lacking an amino or hydroxyl group showed any significant antitubercular effect. However, among those compounds which did show some activity the effect was not of the same order of magnitude as that of isonicotinic hydrazide.

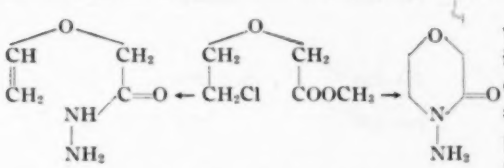
In consideration of the report by Offe, Siefken, and Domagk (8) that cyclic compounds such as pyridazones, pyrazolones and phthalazones occasionally show some tuberculostatic activity, we attempted to make representative examples of substances which can be considered as cyclic hydrazides, as given in Table IV. The pyrazolone was made in the usual manner by treatment of the corresponding β-keto ester with hydrazine. The oxazine derivative was obtained from treatment of β-chloroethoxyacetate with hydrazine and as evidence that the product was not the isomeric vinyl ether the material was found to be

* Received October 16, 1952, from the Warner-Chilcott Research Laboratories, 113 West 18th Street, New York 11, New York.

† Microanalyses were carried out under the supervision of Dr. F. A. Buehler. The activity data on the compounds herein discussed were obtained by Mr. Benjamin S. Schwartz, Mr. Lawrence Landis, and Mrs. Dorothy Kley Crichlow to whom we are greatly indebted. We also wish to acknowledge the technical assistance of Mr. Gordon Kane.

TABLE I.—ALKYL HYDRAZIDES

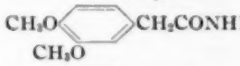
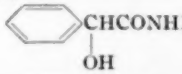
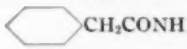
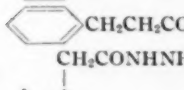
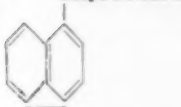
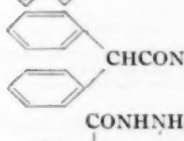
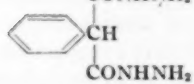
No.	Compound	M. P.	Ref.	Activity
1	n-$C_3H_7CONHNH_2$	39–40°	(13)	–
2	$CH_3CH{=}CHCONHNH_2$	Syrup	(14)	–
3	$CH_2{=}CH(CH_2)_8CONHNH_2$	82–84°	(15)	–
4	$CH_2CONHNH_2$ | CN	108–109°	(16)	–
5	$CH_2CONHCHCONHNH_2$ | CN	268–269°	*E*	–
6	$CONHNH_2$ | $CONHNH_2$	241°	(17)	–
7	$CONHNH_2$ | CH_2 | $CONHNH_2$	152–153°	(17)	–



unchanged on heating and with dilute aqueous acid, which conditions would destroy a vinyl ether (9). Nothing noteworthy was involved in the preparation of the other cyclic hydrazides. In none of these several types of cyclic hydrazides was any usefully significant antitubercular activity encountered, although some closely related derivatives of some of the substances (for example, the 1-isoamyloxy-3-phenyl homolog of phthalhydrazide) have been reported (5) to have appreciable activity.

Several hydrazine derivatives of (substituted) benzenesulfonic acids were prepared, as listed in Table V (Nos. 38–43), and these too were devoid of any significant activity except for possibly weak activity in N-benzenesulfonyl-N′-phenyl-

TABLE II.—ARALKYL HYDRAZIDES

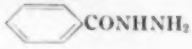
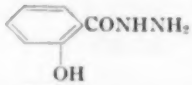
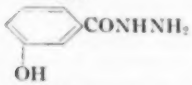
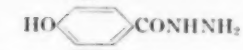
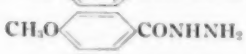
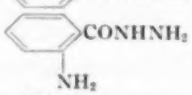
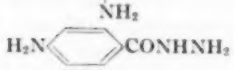
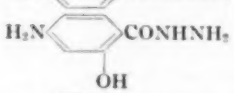
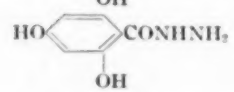
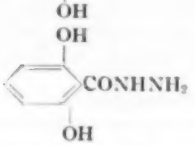
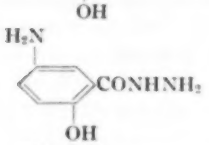
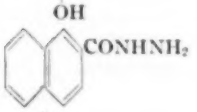
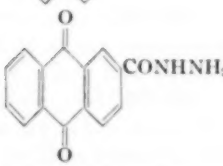
No.	Compound	M. P.	Ref.	Activity
8		106–107°[a]	(18)	–
9		132–133°	(2, 19)	–[b]
10		122–123°	*E*[c]	–
11		102–103°	(20)	–
12		168–169°	*E*[c]	–
13		129–131°	(21)	–[b]
14		179–180°	*E*	–

[a] The reported m. p. was 115–116°. Our compound gave a satisfactory analysis; calcd. for $C_{10}H_{14}N_2O_3$: C, 57.14; H, 6.71. Found: C, 57.17; H, 6.52.

[b] Also reported by Bernstein (2) to be ineffective.

[c] This compound may have been investigated by Buu-Hoi, *et al.* (6); they named it (1-naphthyl)acetic hydrazide but gave the structural formula for 1-naphthoic hydrazide. Their compound was inhibitory at a concentration of 10^{-5} against the BCG strain of *Mycobacterium tuberculosis* by their method of evaluation; isonicotinic hydrazide was inhibitory at a concentration of 10^{-6}.

TABLE III.—ARYL HYDRAZIDES

No.	Compound	M. P.	Ref.	Activity
15		112–114°	(2, 22)	—[a]
16		91– 92°	*E*	–
17		148–149°	(23)	±
18		152–154°	(23)	±
19		262–263°	(2, 6, 23)	[a,b,c]
20		139–140°	(2, 24)	[a,b,c]
21		119–120°	(2, 25)	[a,c]
22		221–223°	(2, 6, 26)	±[a]
23		199–200°	(27)	±
24		234°	(28)	±
25		212–213°	*E*	–
26		180–182°	*E*	–
27		149–151°	(29)	–
28		233–235°	*E*	–

[a] Also reported by Bernstein (2) to lack appreciable activity.
[b] Reported by Buu-Hoi (6) to be inhibitory only at a concentration of 10^{-4} or greater against the BCG strain of *Mycobacterium tuberculosis* by their method of evaluation; isonicotinic hydrazide was inhibitory at a concentration of 10^{-6}.
[c] Our evaluation of this compound was stopped on receipt of the publication of Bernstein (2).

hydrazine. Despite controversial reports (7, 10) of the activity of hydroxamic acids, we prepared a few of these (Nos. 44–48) and found them to be inactive. Since from their structural features one might reasonably expect hydroxamic acids to form more effectively organometallic complexes than would hydrazides (actually hydroxamic acids can be purified via their metal complexes), the antitubercular inactivity of the hydroxamic acids leads one to doubt that the ability to form a copper chelate complex is of the importance in an antitubercular drug that has been suggested (11, 12).

EXPERIMENTAL

The new carboxylic hydrazides were prepared by

the general procedure of refluxing the methyl or ethyl ester of the acid with an excess of 100% hydrazine hydrate for a number of hours and then recrystallizing the resultant material one or more times from an appropriate solvent. Accordingly, it is considered unnecessary to give detailed procedures for each compound and only the pertinent facts are presented in outline form.

Acetamidocyanoacetic Hydrazide.—The ethyl ester and hydrazine hydrate were mixed at room temperature and underwent an exothermic reaction that was controlled by external cooling to ca. 25°. After several minutes the product crystallized and was recrystallized from water.

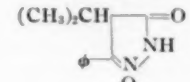
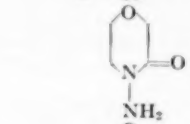
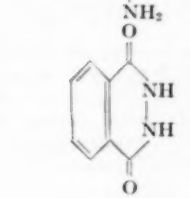
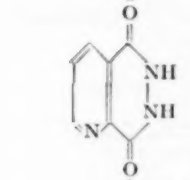
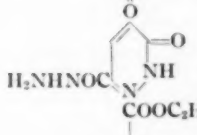
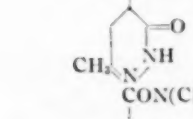
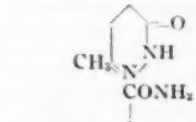
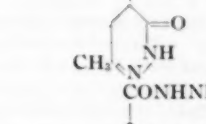
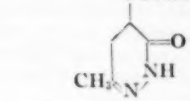
Anal.—Calcd. for $C_5H_8N_4O_2$: C, 38.47; H, 5.17. Found: C, 38.80; H, 5.13.

Cyclohexylacetic Hydrazide.—The methyl ester was used, refluxed eight hours (internal temperature 120°), product recrystallized from benzene.

Anal.—Calcd. for $C_8H_{16}N_2O$: C, 61.50; H, 10.33. Found: C, 61.79; H, 9.96.

(1-Naphthyl)acetic Hydrazide.—The methyl ester was used, refluxed one-half hour, product recrystallized from alcohol.

TABLE IV.—CYCLIC HYDRAZIDES

No.	Compound	M. P.	Ref.	Activity
29		182–183°	*E*	–
30		63– 64°	*E*[a]	–
31		340°	(30)	±[b]
32		308–310°	(31)	–[c]
33		>300°	*E*	–
34		78– 80°	(32)	–
35		183–184°	*E*	–
36		181–182°	*E*	–
37		151–153°	(32)	–

[a] This compound was prepared by Dr. Robert I. Meltzer.
[b] Also reported by Buu-Hoi, *et al.* (3, 4), to have some activity, by their test method.
[c] Also reported by Buu-Hoi, *et al.* (5), to lack significant activity, by their test method.

TABLE V.—MISCELLANEOUS NITROGENOUS DERIVATIVES

No.	Compound	M. P.	Ref.	Activity
38		98–100°	(33)	–
39		157–159°	(34)	±
40		185°	(35)[a]	–
41		221–222° (dec.)	(36)	–
42		234° (dec.)	(36)	–
43		194°	(37)	–
44		171°	*E*	–[b,c]
45		167°	*E*	–[b]
46		129–130°	(38)	–
47		199° (dec.)	(39)	–
48		170° (dec.)	(39)	–
49		116–118°	(40)	–

[a] The deacetylated compound has been reported by Bernstein, *et al.* (2), to be inactive.
[b] The hydrochloride was also reported by Gardner, *et al.* (7), to lack activity.
[c] Also reported by Bernstein, *et al.* (2), to be inactive.

Anal.—Calcd. for $C_{12}H_{12}N_2O$: C, 72.00; H, 6.04. Found: C, 72.20; H, 5.82.

Phenylmalonic Dihydrazide.—The methyl ester was used, refluxed four hours, recrystallized from water.

Anal.—Calcd. for $C_9H_{12}N_4O_2$: C, 51.91; H, 5.81. Found: C, 51.80; H, 5.75.

4-*iso*-Propylbenzoic Hydrazide.—The methyl ester was used, refluxed sixteen hours, product recrystallized from benzene-hexane mixture.

Anal.—Calcd. for $C_{10}H_{14}N_2O$: C, 67.39; H, 7.92. Found: C, 66.92; H, 7.36.

2,6-Dihydroxybenzoic (γ-Resorcylic) Hydrazide.—The methyl ester was used in methanol as a diluent; hydrazinolysis was exothermic, product recrystallized once from alcohol.

Anal.—Calcd. for $C_7H_8N_2O_3$: C, 49.99; H, 4.79; N, 16.66. Found: C, 50.29; H, 4.95; N, 16.05.

3-Amino-6-hydroxybenzoic Hydrazide. — The methyl ester was used, heated on steam bath for one hour, product recrystallized from water.

Anal.—Calcd. for $C_7H_9N_3O_2$: C, 50.29; H, 5.43. Found: C, 50.83; H, 5.46.

Anthraquinone-2-carboxylic Hydrazide. — The methyl ester was used, heated on steam bath for two hours using a little alcohol as a diluent, product recrystallized from a dioxane-hexane mixture.

Anal.—Calcd. for $C_{15}H_{10}N_2O_3$: C, 67.66; H, 3.79. Found: C, 67.70; H, 3.71.

4-Isopropyl-5-phenyl-3-pyrazolone.—A mixture of ethyl α-benzoylisovalerate (13.5 Gm., 0.058 mole) and 85% hydrazine hydrate (4.4 Gm., 0.07 mole) was heated three hours on the steam bath, then refluxed one hour and cooled. The crystalline mass was broken up and washed with water and then with hexane and recrystallized from a 1:1 mixture of ethanol and hexane to give 5.5 Gm. of product.

Anal.—Calcd. for $C_{12}H_{14}N_2O$: C, 71.23; H, 6.97. Found: C, 71.39; H, 6.90.

3-Hydroxy-4-amino-5,6-dihydro-1:4-oxazine.—A mixture of methyl β-chloroethoxyacetate (9 Gm.) (41) and 100% hydrazine hydrate (5 Gm.) was heated for two hours on a hot plate and then cooled to yield a gummy precipitate of hydrazine hydrochloride. The supernatant liquid was decanted and crystallized by the addition of hexane. After recrystallization from 4:1 toluene-hexane the product weighed 3.7 Gm.

Anal.—Calcd. for $C_4H_8N_2O_2$: C, 41.38; H, 6.94. Found: C, 41.72; H, 6.83.

One-tenth gram of the material was allowed to stand at room temperature for two hours in 10 cc. of 1 *N* hydrochloric acid and then the solution was evaporated to dryness on the steam bath. Crystallization of the residue from toluene-hexane gave unchanged starting material as evidenced by its melting point and mixed melting point.

3-Pyridazonyl-6-carboxhydrazide.—A mixture of ethyl 3-pyridazonyl-6-carboxylate (42) (3.4 Gm., 0.02 mole) and 85% hydrazine hydrate (2.4 Gm., 0.04 mole) in 10 cc. ethanol was warmed on the steam bath a few minutes, then cooled and filtered. The product weighed 1.8 Gm. after recrystallization from ethanol.

Anal.—Calcd. for $C_5H_6N_4O_2$: C, 38.96; H, 3.92. Found: C, 39.21; H, 4.09.

4-Dimethylcarbamido-6-methyl-4,5-dihydro-3-pyridazone.—Five grams of ethyl 6-methyl-4,5-dihydro-3-pyridazonyl-4-carboxylate (32) was kept molten at 200° while gaseous dimethylamine was passed through it for two hours. The cooled reaction product was recrystallized from alcohol, with charcoaling.

Anal.—Calcd. for $C_8H_{13}N_3O_2$: C, 52.44; H, 7.11. Found: C, 52.39; H, 7.31.

4-Carbamido-6-methyl-4,5-dihydro-3-pyridazone.—A mixture of ethyl 6-methyl-4,5-dihydro-3-pyridazonyl-4-carboxylate (32), (5.3 Gm., 0.03 mole) and 50 cc. of concentrated aqueous ammonia was shaken thoroughly and allowed to stand at room temperature for three hours. The clear solution was evaporated under vacuum to a crystalline residue which was recrystallized from methanol, with charcoaling, to give 1.9 Gm. of product.

Anal.—Calcd. for $C_6H_9N_3O_2$: C, 46.44; H, 5.85. Found: C, 46.52; H, 5.86.

4-Pyridyl Hydroxamic Acid.—Activity data on this compound were reported by Bernstein, *et al.* (2), and activity data plus physical constants on its hydrochloride were reported by Gardner, *et al.* (7), but we could not find a description of the free base in the literature. It was prepared from ethyl isonicotinate and hydroxylamine in much the same manner as described for *p*-aminosalicylhydroxamic acid (7). The reaction mixture was evaporated to dryness under vacuum and the residue was fractionally crystallized from ethanol-ether.

Anal.—Calcd. for $C_6H_6N_2O_2$: C, 52.19; H, 4.38. Found: C, 52.01; H, 4.31.

3-Pyridyl Hydroxamic Acid.—Activity data and physical constants on the hydrochloride were reported by Gardner, *et al.* (7), and, although the free base was mentioned by Frommel, *et al.* (43), it was not characterized. We prepared it analogously to the 4-isomer except that it was fractionally crystallized from ethanol-hexane.

Anal.—Calcd. for $C_6H_6N_2O_2$: C, 52.19; H, 4.38; N, 20.29. Found: C, 52.24; H, 4.23; N, 20.00.

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Semiquantitative Assay of a Neomycin-Fungistat Ointment*

By MICHAEL SIEGEL

A simplified, semiquantitative assay method for ointments containing combinations of antibiotics, antibacterials, or antimycotics is described. Dilutions are made directly into appropriate media without extraction, and test organisms are used that are sensitive to one antibiotic and not to the other.

THE TOPICAL use of neomycin for the treatment of bacterial skin infections has recently attracted wide interest (1–3). However, both Forbes (4) and Livingood, *et al.* (5), have noted the overgrowth of *Candida albicans* following the extended use of neomycin. To counteract this difficulty we have developed a new ointment, Neomystat, combining the fungistat propyl parahydroxybenzoate (propyl paraben U. S. P.) with neomycin sulfate.

As a control assay on this ointment, to check the amounts of neomycin and propyl paraben present, we have developed the following adaptation of the agar-streak assay method of Waksman and Reilly (6). This test seems a desirable addition to assay methods because of the reproducibility of results, its capacity to semiquantitatively assay more than one substance at once, the elimination of extraction procedures and standardization of test cultures, and the possibility of its use with other antibacterial combinations.

EXPERIMENTAL

Medium.—For preliminary growth of the organisms trypticase soy broth of the Baltimore Biological Laboratories (trypticase 1.7%, phytone 0.3%, sodium chloride 0.5%, dipotassium phosphate 0.25%, and dextrose 0.25%) is used, at *p*H 7.3. It is autoclaved at 15 lb. for twenty minutes, in test tubes 150 mm. long with an outer diameter of 18 mm.

For plates and dilutions extract agar (BBL) (peptones 1.0%, beef extract 0.5%, sodium chloride 0.5%, agar 1.5%) is used at *p*H 7.3, autoclaved twenty minutes at 15 lb.

Organisms.—*Escherichia coli* (ATCC 9637) is used to assay the amount of neomycin present, and *Candida albicans* (ATCC 10231) to assay the fungistat. Stock nutrient agar cultures are transferred to the trypticase soy broth and incubated for twenty-four hours at 33°. They are then retransferred in the same medium, reincubated at 33°, and are ready for use in twenty-four hours.

Preparation of Dilutions.—The neomycin and propyl paraben are not extracted from the ointment base. A weighed amount of the ointment is added directly to a flask containing a measured amount of melted extract agar at 55 to 60°. This is warm enough to melt the ointment completely. The mixture is thoroughly agitated and dilutions made by pipette into other measured quantities of melted extract agar. Dilutions are made so that one is at the estimated point of inhibition for each substance being assayed, with at least one dilution above this concentration and two below it. Each dilution should be made in duplicate. It is very important that dilutions be thoroughly agitated before and during pipetting, and before plating. Our dilutions are made in 250-ml. flasks, and set up so that the total volume is about 50 ml.

Preparation of Plates.—About 10 ml. of each dilution are poured into sterile 10 × 100-mm. Petri dishes in duplicate. The plates are allowed to cool at least twenty minutes before use. They are marked in half, one side for the *E. coli* and the other for the *C. albicans*. The plates are streaked as described by Waksman and Reilly (6). A bent wire needle that will produce a streak about 1 cm. wide is used. The wire is flamed, dipped into the broth culture, and three discrete streaks are made without recharging or flaming the needle. The needle is reflamed between plates, and between organisms. Plates are inverted and incubated eighteen hours at 33°.

RESULTS

The growth of the organisms is compared to their growth on control plates. Plates containing extract agar diluted with petrolatum to give dilutions similar to the ointment dilutions themselves, and then streaked as above, gave the same results as control plates using undiluted extract agar. A typical assay is shown in Table I.

The accuracy of our method was checked against the standard method, which includes extraction of the neomycin sulfate from the ointment (7). The ointment is dissolved in a 50/50 mixture of ether-petroleum ether and the neomycin removed by washing with water three times, and combining these water extracts. Using the method described above, dilutions in agar of unextracted ointment and dilutions of water extracts from the same ointment gave the results shown in Table II. There is no apparent difference.

A comparison between this method and the standard method can be seen in Table III. The latter involves the assay of the water extracts by the paper disk agar diffusion method, using the single inoculated agar layer-standard response curve technique.

* Received October 8, 1952, from the Ethical Research Laboratories, Warren, Pa.

TABLE I.—TYPICAL GROWTH READINGS OF NEOMYCIN-PROPYL PARABEN OINTMENT ASSAY[a]

Dilution	Each Ml. Agar Contains: Propyl Paraben, Mcg.	Neomycin, Units	18 Hr. Growth on 4 Plates: C. Albicans	E. Coli
1	120	12.6	0, 0, 0, 0	0, 0, 0, 0
2	100	10.5	0, 0, 0, 0	0, 0, 0, 0
3	80	8.4	1, 2, 1, 1	0, 0, 0, 0
4	12	1.25	2, 2½, 2, 3	0, 0, 0, 0
5	9.5	1.0	3, 3, 3, 3	0, 0, 0, 0
6	7.1	0.75	3, 3, 3, 3	1, 1, 1½, 1
7	3.3	0.35	3, 3, 3, 3	3, 3, 3, 3
Control	0	0	3, 3, 3, 3	3, 3, 3, 3

[a] Two plates are poured from each duplicate dilution. Readings go from 0 (no growth) through 3 (for the number of streaks showing growth). Each gram of ointment contained 5 mg. (1,050 units) of neomycin and 10 mg. of propylparaben.

TABLE II.—COMPARISON BETWEEN GROWTH OF E. COLI ON AGAR DILUTIONS (1) FROM WATER EXTRACTS OF NEOMYCIN FROM AN OINTMENT, AND (2) FROM UNEXTRACTED OINTMENT[a]

(1) Extraction Dilutions in Units Neomycin per Ml.	Growth	(2) Ointment Dilutions in Units Neomycin per Ml.	Growth
1.6	0, 0, 0, 0	1.6	0, 0, 0, 0
1.3	0, 0, 0, 0	1.2	0, 0, 0, 0
1.0	0, 0, ½, 0	1.0	0, 0, 0, 0
0.85	½, ½, ½, 1	0.75	1, 1, ½, 1
0.65	1, 2, 1, 2	0.6	2, 1½, 1, 1
0.35	3, 3, 3, 3	0.3	3, 3, 3, 3
0.14	3, 3, 3, 3	0.1	3, 3, 3, 3
Water	3, 3, 3, 3	Petrolatum	3, 3, 3, 3

[a] Two plates are poured from each duplicate dilution. Readings go from 0 (no growth) through 3 (for the number of streaks showing growth). Each gram of ointment contained 5 mg. (1,050 units) of neomycin and 10 mg. of propylparaben.

TABLE III.—COMPARISON OF THIS METHOD WITH THE STANDARD ASSAY[a]

	This Method: Range	This Method: Ointment Assay	Standard Method: Ointment Assay
Ointment No. 1	950–1,160	1,050	1,100
Ointment No. 2	900–1,180	1,050	1,030

[a] Measurements are in units of neomycin per gram of ointment. Dilutions under "Range" are those just above and just below the dilution estimated as containing 1.0 unit of neomycin per ml. agar (i. e., 1,050 units per Gm. ointment).

DISCUSSION

Work in our laboratories has shown that on extract agar at 33° for eighteen hours 100 mcg. of propyl paraben per ml. will completely inhibit the growth of *Candida albicans*. One unit of neomycin per ml. of agar is the inhibitory point of *Escherichia coli* by definition (8). Our dilutions are therefore made to include and encompass both these points (see Table I). Using neomycin alone in petrolatum there is no inhibition of *C. albicans* up through a concentration of 25 units per ml. Conversely, the paraben alone in petrolatum up to a concentration of 200 mcg./ml. has no effect on *E. coli*. It is obviously important to use test organisms that avoid an overlapping of the inhibitory effects of the substances being assayed.

We have found partial inhibition of *E. coli* by neomycin begins at a concentration between 0.4 and 0.5 unit/ml.; and partial inhibition of *C. albicans* by propyl paraben as low as 12 mcg./ml. To obtain full growth of both organisms for comparison, at least one dilution must be below those points.

Theoretically, a concentration of one unit of neomycin per ml. can be found anywhere between the dilution that just shows growth and the first one that inhibits *E. coli*, up to and including the latter dilution. Actually, since this method is used for control work and we are therefore able to pick our dilutions, plus the fact that there is an almost straight line decrease of growth from 0.5 unit of neomycin up to 1.0 unit, the assay becomes semi-quantitative. Use of the dilution above the first completely inhibitory one plus the one below gives a range in which the unitage dilution should fall. This can be seen in Table III.

Other than the appearance of a good, turbid suspension, no attempt is made to standardize the growth of the test organisms in the broth cultures. Since maximum growth is determined by the growth on control plates for each test, standardization of the cultures is not necessary. Another variation occurs in the amount of inoculum, which will depend on culture viscosity and surface tension, and the age of the inoculating wire (the rougher surfaces of older wires will hold more inoculum than newer ones). Here again, the use of individual controls and the same wire in individual tests, as well as diluting in duplicate and plating in duplicate, make this variation unimportant.

The use of different amounts of the same dilutions per plate, from 5 ml. up to 25 ml., made no difference in the results. The concentration of the antibiotic and fungistat per ml., or perhaps even per cm.^{2}, is the critical factor.

This method is less precise than the various modifications of the agar diffusion techniques, such as standard response curve or double dose (7), but it is rapid and direct, results are readily reproducible,

it requires less preliminary preparation and eliminates extraction procedures, and permits the assay of more than one substance at once, regardless of water solubility. Note that although neomycin is very water soluble, the solubility of propyl paraben is only 5 mcg. per ml. water while its inhibitory concentration is 20 times that; however, this in no way interferes with the results.

To assay other combinations, particularly antibiotic combinations, it is merely necessary to find test organisms, so that one is sensitive to one antibiotic and not to the other, and vice versa. We are now working on this in our laboratories.

SUMMARY

An adaptation of the Waksman-Reilly (6) agar streak assay technique is described in which combinations of antibiotics and other bacteriostats and fungistats may be readily assayed semiquantitatively without preliminary separation, without regard to water solubilities, and without the need for extraction from ointment bases.

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A Comparative Study of the Effect of Various Solutions on the Calcium Depletion of The Rat Tooth Using Radioactive Calcium*,†

By CHIEKO URAKAMI, CHI-TAO WANG, JOHN B. DATA, and J. E. CHRISTIAN

Calcium in rats' teeth was labeled with radioactive Ca-45 by feeding the animals a calcium-free diet and administering calcium as calcium-45 levulinate biweekly for eight weeks starting with a two weeks' old animal. The radioactive teeth were removed and the calcium depletion which resulted from immersion of the teeth in various liquids for twenty-four hours was determined by tracer methods. The liquids tested were distilled water, commercial mouthwashes, citric and tartaric acid solutions, beverages, milk, and human saliva.

IN AN EARLIER communication from this laboratory (1) a method was reported for the determination of the effect of various solutions on phosphorus depletion of rat teeth using radioactive techniques. In a continuation of this earlier work the original method has been improved by the introduction of the following changes: (*a*) the tooth was suspended into the test solution from a glass holder, thus keeping the broken end of the tooth out of the solution; (*b*) the teeth were screened and those which ground to 11 mm. in length and weighed 0.034–0.039 Gm. were selected in order to expose a reasonably uniform area of tooth surface to the test solution; (*c*) the teeth were removed without prior boiling of the rat's head in water to avoid contamination, and (*d*) calculation of the percentage depletion is based on total radioactivity of the tooth tested rather than on total radioactivity determined on one of the four incisor teeth from the same rat.

In the earlier technique, radioactive phosphorus was used in preference to radioactive calcium because of its ready availability and for its presence as a component of tooth structure. While radioactive phosphorus has certain limitations for teeth studies due to its inherent half-life, it serves, nevertheless, as a useful tool for a rapid, semiquantitative measure of corrosive action in minute quantities of certain solutions on rat teeth. As radioactive calcium became available at a reasonable price, it offered a direct method for the measurement of corrosive action since calcium depletion, to some extent, could be made to represent a permanent part of the tooth structure.

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† The isotopes used in this research were allocated by the United States Atomic Energy Commission.

The object of the present study was to compare calcium depletion[1] of several solutions on teeth obtained from rats made radioactive by two different methods. These consist of injecting radioactive calcium intraperitoneally twenty-four hours prior to the removal of the teeth (designated short-term), and by administering biweekly for eight weeks, radioactive calcium intraperitoneally to two-week old rats maintained on a low calcium diet (referred to as long-term). The results of these experiments were to serve in the selection of the most plausible and significant method to evaluate additional solutions.

EXPERIMENTAL

The Animal Colony.—It was considered necessary to control as well as possible the following factors: (*a*) the age of the rat, (*b*) the weight of the rat, (*c*) the calcification of the incisor with a maximum amount of radiocalcium, and (*d*) the size and weight of the incisor. The first two factors were controlled by breeding of the animals to be used. The third factor was taken care of by feeding a special calcium deficient diet and frequent administration of an excess amount of calcium-45 labeled calcium levulinate. The last factor was controlled by the careful screening of incisors according to their size and weight.

The diet consisted of the following ingredients (2): Weber Flour Mills Company's Eagle Brand Cracked Wheat (calcium content 0.081%) and Sunshine Dairy Farm's Milk Powder (calcium content 1.8% on dry basis) mixed in the ratio of 5:1. Sodium chloride U. S. P., was added to the extent of 2% of the weight of the wheat. Cod liver oil was used once every week for supplement. The food and distilled water was taken by the rats *ad lib.*

For the long-term experiments two-week old rats were injected with radiocalcium levulinate until they reached ten weeks of age. The course of injections was as follows: The young rat at two weeks of age was given a 10-mg. intraperitoneal injection of calcium levulinate. From that time on two 10-mg. injections were given every week at three and one-half day intervals until the age of ten weeks. A total of 15 injections, amounting to 150 mg. of calcium levulinate, was given. Three days after the last injection the animal was sacrificed with chloroform.

In the short-term experiments, rats ten weeks of age were given one injection of 0.25 Gm./Kg. of body weight. The total activity was about 5 microcuries. Twenty-four hours after injection the rats were sacrificed.

Counting Technique.—Considerable experimentation was done to develop a satisfactory counting technique for determining the radioactivity of the solutions tested. This problem was more difficult than the usual counting techniques since it was necessary to evaporate to dryness as much as 5 ml. of testing liquid and have the residue uniformly distributed in the bottom of sample holders $1^1/_4$ in. diameter and $^1/_8$ in. deep. This was necessary since calcium-45 is a weak β-emitter and since maximum activity values were important in obtaining statistically significant results.

After carefully checking many known samples, the following procedure was found to yield reproducible results and at the same time give maximum activities. The solution which was to be counted was placed in a 30-ml. beaker which had been rinsed previously with a 1% Aerosol wetting agent and dried. A piece of muslin $1^1/_4$ in. in diameter was lowered into the beaker in such a manner that it lay flat on the bottom. The contents of the beaker were then evaporated slowly to dryness under an infrared lamp. The muslin was then removed carefully from the beaker to an aluminum counting cup ($1^1/_4$ in. diameter and $^1/_8$ in. deep) which had been treated previously with a few drops of shellac. The cup was placed in a Model D-46 "Q-Gas" counter (Nuclear Instrument and Chemical Corporation) and the activity determined. The usual correction factors for resolution, background, and self absorption were applied to the values obtained to give corrected activities. Using adequate counting time it was found that this procedure gave values correct to 1.5% error or less and the over-all efficiency of detection was comparable to other methods. Ninety-six per cent of the total activity when compared to other methods was found to be detected with great consistency.

Procedures for the Determination of the Calcium Depletion of Teeth After Exposure to Various Solutions.—The animals were sacrificed by means of chloroform and a part of the jaw bone and tooth was cut off with a pair of scissors back far enough to give sufficient tooth. The bony structure attached to the tooth was removed by cutting with a sharp, thin cutting tool and then separating the tooth from the bone. Each tooth was then cleaned by rubbing it with a cloth moistened with water. After allowing the tooth to air dry for six to eight hours, the broken end of the tooth was ground with a mechanical grinder to give a tooth 11 mm. long and weighing 0.034–0.039 Gm. Each tooth, selected as described above, was next placed in a cloth sack fastened on a glass stirrer, and stirred for twenty-four hours in running tap water. The stirring was continued in slowly changing distilled water for another ninety-two hours. This was done to remove all water-soluble calcium from the teeth.

Each tooth after washing was placed in a glass holder and held in position with wax (Fig. 1). This was achieved by first setting the tooth in partly solidified wax and then pushing the tooth into position with a metal pattern designed such that it acted as a measure for reproducing the desired length of protruding tooth (7.5 mm.). The wax was smoothed about the surface and around the tooth with a spatula which was preheated sufficiently to liquefy the wax. After the wax solidified (one to two hours) the tooth was cleaned with a cloth moistened with acetone and then rechecked with the metal pattern for correct exposed length.

Three millimeters of test solution was pipetted into a 5-ml. volumetric flask. The glass holder containing the tooth was introduced into the neck of the volumetric flask, and the tooth adjusted to such a depth in the solution that the end of the

[1] The term "depletion" is used freely to designate the quantity of calcium removed from the tooth without reference to the chemical form in which it may exist or be removed from the tooth or by what mechanism it is removed.

glass holder was at the same level as the bottom of the meniscus of the solution (Fig. 1). The flask was placed in a 37° constant temperature water bath for twenty-four hours. At the end of this time the holder was removed and the tooth washed with sufficient distilled water from a medicine dropper directly into the flask to make the volume 5 ml. This solution was labeled "A."

The tooth was then removed from the holder. Any wax adhering to its surface was wiped off with a cloth moistened with acetone. The broken end of the tooth was ground back with a mechanical grinder to the level it was exposed to the solution. The pattern was again used as a guide to the proper amount to grind off. The residual tooth was placed into a 5-ml. volumetric flask containing 1 ml. of concentrated hydrochloric acid which slowly dissolves the tooth. Solution of the tooth may be hastened by placing the flask in a water bath at 37°. When the tooth was dissolved, sufficient water was added to the flask to make 5 ml. This solution was labeled "B."

The activities of solutions "A" and "B" were determined using the technique described and the percentage of depletion of calcium was calculated as follows: If "A" is the radioactivity in c. p. m. of the test solution and "B" is the radioactivity in c. p. m. of the residual solution, the percentage of calcium depletion due to the test solution is $A/(A + B) \times 100$.

Results.—In order to ascertain whether all of the water-soluble calcium was removed during the washing process, 53 short-term teeth and 60 long-term teeth were subjected to the above procedure using triple distilled water as the test solution. The average value obtained was 0.18%. This represents such a small amount of depletion that it is disregarded in the calculation of depletion in the various test solutions.

Tables I and II summarize the results obtained from the study of the calcium depletion of rats' teeth (short-term and long-term, respectively) after being subjected to various solutions held at 37° for twenty-four hours *in vitro*

TABLE I.—PER CENT CALCIUM DEPLETION OF RATS' TEETH AFTER IMMERSION IN VARIOUS SOLUTIONS (DETERMINATION MADE ON "SHORT-TERM" TEETH[a])

Solution	No. of Teeth	Average Depletion, %	Deviation[b]
Triple distilled water	53	0.16	...
Commercial mouthwash No. 1	18	0.24	± 0.11
Citric acid (0.001 *M*)	8	0.62	± 0.18
Citric acid (0.005 *M*)	4	1.36	± 0.37
Citric acid (0.01 *M*)	8	2.30	± 0.56
Citric acid (0.05 *M*)	8	5.20	± 1.1
Citric acid (0.06 *M*)	6	7.40	± 1.6
Citric acid (0.07 *M*)	5	20.3	± 7.8
Citric acid (0.08 *M*)	5	39.9	±13.9
Citric acid (0.09 *M*)	5	76.4	±21.2
Citric acid (0.1 *M*)	5	89.8	±24.4

[a] Teeth made radioactive and removed twenty-four hours after a single injection of radiocalcium levulinate.

[b] $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

DISCUSSION

An evaluation of the data compiled from both short-and long-term teeth studies led us to conclude

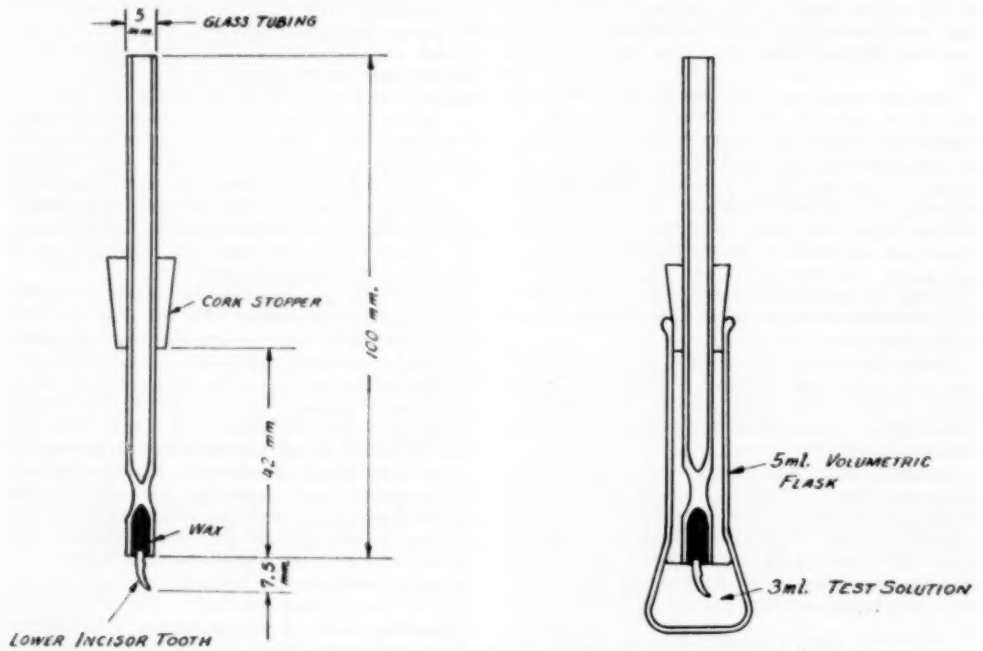

Fig. 1.—Procedure for the determination of calcium depletion in rats' teeth.

from these two sets of experiments that the results obtained from the long-term work were more significant for evaluating the corrosive action of various solutions on rat teeth than from the short-term work. This conclusion is based on the most plausible interpretation of the experimental data and in consideration of the experimental conditions. As the tooth develops in the growing young rat in the presence of controlled quantities of radioactive calcium in the body, it is very likely that the Ca-45 laid down as a permanent part of the tooth structure is distributed in the tooth in a high degree of homogeneity. A radioautograph of a cross and longitudinal section would serve as a means to confirm this. The removal of some labile[2] Ca-45 by a leaching process, as explained under "Experimental" above, for both short- and long-term teeth, gave a labeled tooth from which no appreciable quantities of Ca-45 is depleted with distilled water.

TABLE II.—PER CENT CALCIUM DEPLETION OF RATS' TEETH AFTER IMMERSION IN VARIOUS SOLUTIONS (DETERMINATION MADE ON "LONG-TERM" TEETH[a])

Solution	No. of Teeth	Average Depletion, %[b]	Deviation
Triple distilled water	60	0.20	...
Commercial mouthwash No. 1	34	2.0	±0.33
Citric acid (0.005 *M*)	15	11.1	±3.2
Citric acid (0.01 *M*)	15	19.1	±2.5
Tartaric acid (0.005 *M*)	7	8.7	±1.7
Tartaric acid (0.01 *M*)	7	13.4	±2.7
Tartaric acid (0.05 *M*)	8	22.0	±1.6
Tartaric acid (0.08 *M*)	7	28.4	±2.7
Tartaric acid (0.1 *M*)	8	29.6	±2.8
Alkaline aromatic solution	15	0.2	...
Whiskey (100 proof)	8	0.0	...
Commercial beverage	8	7.7	±1.8
Commercial mouthwash No. 2	8	0.0	...
Fresh skimmed milk	24	0.25	...
Human saliva	8	0.29	...

[a] Teeth made radioactive by injection of animal with two 10-mg. radiocalcium levulinate injections per week for eight weeks starting when the animal was two weeks of age. Twenty-four hours after the last injection the teeth were removed.

[b] The activity of the residual teeth varied from 20,000 c. p. m. to 40,000 c. p. m. and that of the test solution from 0 c. p. m. to 1,000 c. p. m. Depletion values less than 0.3% are not considered accurate as the actual value may range from 0 to 0.3%. This is because the activity of the test substance in these instances is just above background and cannot be accurately determined. These values are important, however, in proving that only a small amount of calcium depletion results.

It is very probable that the largest concentration of Ca-45 in short-term teeth is distributed in the innermost part of the tooth, the growing part, and it is very likely labile in view of the short interval which exists between the time of the injection with radioactive calcium and the removal of the labeled tooth; that is, the time interval does not permit a considerable amount of permanent deposition. A comparison of the experimental data obtained from short- and long-term experiments for the same test solution indicates a greater percentage of calcium depletion for long-term teeth than short-term. The only variable factor in each of these similar experiments is the nature of the labeled tooth. In long-term experiments there is no question that the total permanent Ca-45 present is in greater quantities than for short-term teeth. The labile quantities of Ca-45 should be relatively constant for both short- and long-term teeth by virtue of the experimental conditions; namely, each type of tooth is extracted from rats having a high concentration of Ca-45 distributed throughout the animal body prior to sacrificing the animal and removal of the tooth.

The corrosive action of the test solution as measured in terms of percentage calcium depletion thus represents both permanent and labile Ca-45. The difference in percentage of Ca-45 depletion for similar solutions in both short- and long-term experiments could logically be interpreted to represent the percentage of calcium depletion of a permanent nature from the tooth. Since a comparative method for evaluating quantitatively the minute effect of various solutions on the corrosive action of the permanent structure of rat tooth is sought, the long-term tooth appears from preliminary studies to be more adaptable for this purpose. It would now be of interest to compare the results of the latter procedure with those obtained from using teeth removed at intervals from rats growing on a low-calcium diet and administering Ca-45 interperitoneally for growing periods greater than eight weeks.

[2] The term "labile" as used in this article implies calcium which has not been deposited as a permanent part of the tooth structure.

SUMMARY

1. A procedure is described for the accurate and convenient determination of the radioactivity of weak solutions of calcium-45. This procedure was found to detect consistently 96% of the activity as determined by other more tedious methods.

2. A procedure is described for the determination *in vitro* of the calcium depletion of rats' teeth after exposure to various solutions. The procedure is based on the deposition of calcium-45 into the teeth *in vitro* by administering calcium-45 levulinate, removing the teeth, immersing them in various solutions and determining the Ca-45 removed as compared to the total present.

3. Using the lower incisors obtained after a series of 16 injections of labeled calcium levulinate to rats over a period of eight weeks, and lower incisors obtained twenty-four hours after a single injection, the percentage calcium depletion after exposure for twenty-four hours was determined for a series of concentrations of citric and tartaric acids, for triple distilled water, two commercial mouthwashes, two beverages, alkaline aromatic solution, fresh skimmed milk, and human saliva.

4. Teeth used after a single injection of the calcium salt give a much greater deviation of results than those in which the calcium is administered over a period of time. The use of the latter is considered to give more significant results. It is noteworthy that the calcium depletion of teeth obtained after multiple injections of Ca ion is uniformly greater than otherwise. This is undoubtedly due to differences in the distribution of the calcium in the teeth.

5. The calcium depletion of teeth increases in direct proportion to the increased concentration of acid; however, the acidity of a solution is not the only factor effecting such depletion.

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Chemical Assays of Ergot and Ergot Fluidextract*

By JOHN W. STRONG and F. A. MAURINA

The present article is in part a review of the development of previously reported chemical assay methods for ergot and ergot fluidextract (1). Certain miscellaneous data, subsequently determined while completing this study, are reported for the first time. The *p*-dimethylaminobenzaldehyde color reaction has been examined and its reliability confirmed. Ergonovine maleate has been found a more stable reference standard than ergotoxine ethanesulfonate. The assay methods have been subjected to further collaborative study, following minor revisions, and the results are reported and discussed.

THIS REPORT describes the development of the N. F. IX chemical assay for ergot and a chemical assay for ergot fluidextract is also discussed. The results of collaborative studies sponsored by the U. S. P. and designed to develop a satisfactory method of assay for ergot were reported in 1947 (2). When it was decided to omit ergot and ergot fluidextract from the U. S. P. XIII and to include them in the N. F. VIII instead, that report was immediately made available to the Committee on National Formulary and in turn was presented at a meeting of the Combined Contact Committee held in Washington, D. C., November 11 and 12, 1946. It was decided that the problem required further study before any official assays could be adopted, and to accomplish this a Subcommittee on the Chemical Assay of Ergot[1] was appointed.

The subcommittee accepted two general recommendations of the Smith (2) study group: that the ergot alkaloids should be determined by chemical methods rather than biological because the former are much simpler than the latter and because biological methods possessed no advantage in accuracy when the two types were compared; and that the assay methods for ergot and ergot fluidextract submitted by this group were the best of several studied and offered the best approach to the development of adequate official assays. The methods were therefore subjected to collaborative study by the subcommittee. Variable assay values and difficulties with the procedure were encountered by the collaborators and it was agreed that further improvement in methods should be attempted by the present authors (3).

A report covering the earlier part of this work has already been published (1). The presentation that follows reviews the experimental work involved and reveals certain heretofore unpublished results.[2]

* Received June 4, 1952, from the Analytical Laboratories of Parke, Davis and Company, Detroit, Mich.

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EXPERIMENTAL DESIGN

Solubility and Stability Studies.—The assay of ergot or ergot fluidextract by a photometric method involves extraction of the alkaloids and their color determination. It might be assumed that inherent errors occur throughout the procedure, but the accuracy of the color test perfected by Allport and Cocking (4), based on the reaction between the ergot alkaloids and *p*-dimethylaminobenzaldehyde, was so generally recognized that attention was

[2] The authors wish to acknowledge the considerable collaborative work performed by members of the subcommittee and especially by Ward J. Rice and his staff.

first directed to determining sources of error and means of eliminating them in the extraction procedure. A fundamental study of the solubility distributions of ergotoxine and ergonovine, in the possible two-phase systems involved, was first undertaken because only a small portion of the desired information was to be found in published reports. Determinations were made by shaking a definite volume of an aqueous solution containing a definite concentration of the pure alkaloidal salt with an equal volume of the desired organic solvent, and applying the *p*-dimethylaminobenzaldehyde color procedure directly to the equilibrated aqueous phase where feasible or to an aqueous extract prepared from either the aqueous or organic phase. Some of the more significant results of this study are listed in Table I. In further preliminary studies stability tests involving ergonovine were performed demonstrating the effects of exposure to light and of *p*H under various potential conditions of assay. Bright daylight was found to cause serious decomposition of the alkaloid during extraction, particularly when dissolved as the free alkaloid in an organic solvent or in alkaline aqueous solution, but exposure to moderate artificial light produced no appreciable loss. In the absence of daylight ergonovine was observed to be reasonably stable dissolved in 1% w/v sulfuric acid or in aqueous solution alkalinized with either sodium bicarbonate or ammonium hydroxide. Considerable decomposition occurred, on the other hand, after prolonged standing in the dark in the presence of strong alkali. In these stability studies the *p*-dimethylaminobenzaldehyde color procedure was again used as the basis of measurement. A third factor possibly affecting stability, that of elevated temperature, was not investigated, and strong heating of solvents in extractions and evaporations was generally avoided in the present work. Corran and Rymill have found the alkaloids of ergot to be stable in the presence of certain hot organic solvents (5), but it is not known to what extent heating might increase the rate of decomposition due to light.

TABLE I.—DISTRIBUTIONS OF ERGOTOXINE AND ERGONOVINE BETWEEN EQUAL VOLUMES OF IMMISCIBLE AQUEOUS AND ORGANIC SOLUTIONS

Alkaloid	Aqueous Solution	Immiscible Organic Solvent	Alkaloid in Organic Phase, %
Ergotoxine	1% w/v H_2SO_4	Chloroform	49
Ergotoxine	1% w/v H_2SO_4	1 part chloroform + 3 parts ether	2
Ergotoxine	1% tartaric acid	1 part chloroform + 3 parts ether	5
Ergotoxine	NH_4OH, *p*H = 8–9	Carbon tetrachloride	99
Ergotoxine	1% NH_3	Carbon tetrachloride	96
Ergonovine	5% $NaHCO_3$	Carbon tetrachloride	2
Ergonovine	5% $NaHCO_3$, sat. NaCl	Ether	69
Ergonovine	NH_4OH, *p*H = 8–9	Carbon tetrachloride	2
Ergonovine	NH_4OH, *p*H = 8–9, sat. NaCl	Ether	99
Ergonovine	1% NH_3	Carbon tetrachloride	1
Ergonovine	1% NH_3, sat. NaCl	Ether	97

Extraction Procedure for Ergot.—In developing a revised assay for ergot the desirability of retaining methods for both total alkaloids and ergonovine was recognized. An initial experiment was carried out designed to indicate, first, an efficient solvent or menstruum for the removal of total alkaloids from ergot powder, and secondly, the number of purification steps that might be necessary in determinations of both total alkaloids and ergonovine. Three solvents were selected for comparison: acetone, chloroform, and chloroform-ether mixture (1, 3). These were alkalinized with ammonia water according to the following formulas: (*a*) 99 parts acetone and 1 part stronger ammonia water; (*b*) 90 parts chloroform and 9 parts methanol and 1 part stronger ammonia water; and (*c*) 94 parts chloroform-ether mixture and 5 parts methanol and 1 part stronger ammonia water. Methanol was included to render the mixtures homogeneous. To check necessary purification steps in the assay for total alkaloids, two types of extraction procedure were studied: a relatively simple direct extraction with dilute acid, referred to as the "single" extraction method; and a longer method involving extraction of an alkaline saturated solution of sodium chloride in water using ether, followed by extraction of the ether with dilute acid, which is designated the "two-way" extraction method. Similarly, in the assay for ergonovine a simple "direct" method was applied in the one case and a longer "two-way" method in the other.

EXPERIMENTAL PROCEDURES

Total Alkaloids.—Shake 20 Gm. of powdered ergot with 200 cc. of menstruum one hr. and filter. In the single extraction method evaporate 100 cc. of the acetone or chloroform filtrates at room temperature to 25 cc. and add 75 cc. of ether. Extract 100 cc. of the acetone-ether or chloroform-ether solution so prepared with four 15-cc. portions of 1% w/v sulfuric acid (10% w/v sulfuric acid in first shake where evaporation step was omitted) using 3 cc. additions of alcohol where necessary to control emulsions, wash each portion with 50 cc. of ether, and dilute the combined extracts to 100 cc. with water. Dilute 10 cc. of this solution to 100 cc. with water. Apply the *p*-dimethylaminobenzaldehyde color test to the dilute solution, comparing with ergotoxine ethanesulfonate as the standard. In the two-way extraction method evaporate 100 cc. of the menstruum, filtered free of undissolved drug, to 25 cc. Add 20 cc. of water saturated with sodium chloride, 0.2 cc. of stronger ammonia water, and 5 cc. of alcohol. Extract with 40 cc., 30 cc., 30 cc., and 30 cc. of ether. Extract the ether with four 15-cc. portions of 1% w/v sulfuric acid, and dilute the aqueous extracts

to 100 cc. with water. Dilute and determine the color as above.

Ergonovine.—Extract 25 cc. of the concentrated solution of total alkaloids prepared above, rendered slightly alkaline with ammonia, with three 15-cc. portions of carbon tetrachloride, and wash each portion with 10 cc. of water. Discard the carbon tetrachloride, and combine the aqueous solutions. To complete the assay by the direct extraction method, acidify the aqueous solution, dilute to 50 cc. with water, and measure the alkaloidal content by the *p*-dimethylaminobenzaldehyde color test, comparing with ergonovine maleate as the standard. To apply the two-way extraction method saturate the combined alkaline extracts prepared above with sodium chloride, and extract with 40 cc., 30 cc., 30 cc., and 30 cc. of ether. Extract the ether with 10 cc., 10 cc., 8 cc., and 8 cc. of 1% w/v sulfuric acid, and dilute the aqueous extracts to 50 cc. with water. Determine the color as above.

The results of this study as applied to a given lot of ergot are shown in Table II. Chloroform proved the most efficient solvent and in the absence of other complicating factors was judged the menstruum of choice. The two-way extraction method in the assay for total alkaloids was not found to possess any advantage over the single extraction method as a purification step, since corresponding values by the two methods agreed substantially in most cases, and it was therefore concluded that the shorter method should be adopted. In the assay for ergonovine, however, results were significantly higher by the direct method than by the two-way method in those cases where chloroform was the original menstruum. The possibility was considered that impurities behaving as alkaloids in the color test were present and that these were only partially removed by the two-way extraction step in the ergonovine assay. Repeated application of the two-way process to the ergonovine extracts was then investigated, but a further drop in assay values did not result. The conclusion was reached that the two-way extraction is a necessary step in the assay for ergonovine, though additional manipulation along the same lines serves no useful purpose. Corrections for nonspecific color or background densities were found necessary in the ergonovine assay using the direct method, but no such corrections were needed when the two-way method was used. Background densities were not significant in any of the determinations of total alkaloids.

Two extraction techniques were compared in the removal of total alkaloids from ergot powder: shaking and percolation. Extractions were equally efficient using either technique where chloroform was the menstruum, as assay values did not vary. Continuous extraction, which involves boiling solvents, was not used. Percolation was finally selected because a cumbersome filtration step, required after shaking, is thereby avoided.

The complete extraction procedure finally adopted for powdered ergot combines the following previously discussed steps: (*a*) percolation using chloroform plus the single extraction method in the determination of total alkaloids; and (*b*) the two-way extraction method in the determination of ergonovine.

Extraction Procedure for Ergot Fluidextract.—The approach taken in the development of a modified procedure for ergot fluidextract closely paralleled that followed in the case of ergot. A single extraction method was compared with a two-way extraction in the assay for total alkaloids, and a direct method with a two-way method in the assay for ergonovine. At first it was thought that the assay for total alkaloids might be relatively simple because no initial percolation step was necessary. However, in this simplified version considerable interference was encountered due to colored impurities and emulsions. These difficulties were overcome by the preliminary removal of acetone-insoluble impurities, a step included in the assay procedure developed by the Smith study group (2). Following this preliminary purification step the procedure found most satisfactory for ergot fluidextract resembled that adopted in the case of ergot: the single extraction method for total alkaloids and the two-way extraction method for ergonovine.

TABLE II.—PERFORMANCE OF MENSTRUUM IN EXTRACTION OF UNIFORM SAMPLE OF POWDERED ERGOT. COMPARISON OF SINGLE AND TWO-WAY EXTRACTION METHODS

Sample	Menstruum	Extraction Method for Total Alkaloids	Total Alkaloids, Mg./Gm.[a]	Ergonovine, Mg./Gm.[b]	
				Direct Method	Two-Way Extraction
1	Acetone	Single extraction	3.08	0.261	0.232
2	Acetone	2-way extraction	3.02	0.223	0.226
3	Chloroform-ether	Single extraction	3.30	0.376	0.378
4	Chloroform-ether	2-way extraction	3.26	0.324	0.308
5	Chloroform	Single extraction	4.31	0.478	0.397
6	Chloroform	2-way extraction	4.16	0.443	0.388

[a] Expressed in terms of ergotoxine ethanesulfonate.
[b] Expressed in terms of ergonovine maleate.

Precautionary Measures.—In addition to the necessary precautions which must be observed with regard to exposure to light, the need for a number of other precautionary measures was noted in the course of this study. In order to minimize possible decomposition it was decided that while performing an assay only the acidified alkaloidal extracts should be stored overnight and that these should be refrigerated during storage. Frequently slight precipitates were observed to settle out of solutions of total alkaloids during storage and these were found to contain material giving the alkaloidal test. Fresh mixing of the solutions before withdrawing aliquots prevented errors due to such precipitation. Another precautionary measure was found necessary to prevent the occurrence of unusually high and variable results for ergonovine with certain samples. On diluting two parts of

the concentrated solution of total alkaloids with three parts of water before applying the extraction procedure for ergonovine, the difficulty apparently disappeared, as the resulting assays were then lower and reproducible. The reason for this phenomenon was not readily apparent. Special treatment has sometimes been necessary in the filtration of acetone-insoluble impurities in the assay of the fluidextract. Shaking with asbestos before filtering was usually helpful, or if the mixture was centrifuged, filtration could be omitted entirely. Emulsions sometimes interfered while extracting with carbon tetrachloride in the assay for ergonovine in ergot fluidextract. In such cases the mixture was allowed to settle thirty minutes. All carbon tetrachloride including the emulsified portion was then drained into the bottom separator and sufficient additional carbon tetrachloride was added to this with subsequent shaking to assure a dispersion of the aqueous phase in the organic. The above precautions were not mentioned in the originally prepared assay directions, but were covered in a final draft.

On two occasions, while conducting the assays, the authors have observed considerable decomposition of total alkaloids and particularly of ergonovine, even though no deviations in procedure could be ascertained and all necessary precautions had presumably been followed. Substitution of fresh reagents for those that had been standing on the shelf corrected the difficulty, whereas with reuse of the old reagents the difficulty persisted. Some form of contamination had evidently entered one or more of these reagents, possibly via the atmosphere, but the identity or source of the contaminant could not readily be determined. While this situation has not been covered in directions to collaborators, these observations are believed worthy of record for the purpose of pointing out that more than ordinary precautions should be exercised in handling reagents used in conjunction with ergot assays.

Colorimetric Step.—The absorption spectra in the visible range for the colors formed by ergotoxine and ergonovine in the reaction with *p*-dimethylaminobenzaldehyde were checked with the Beckman Quartz Spectrophotometer (Fig. 1). It will be noted that the absorption curves are broad and that the region of peak absorption extends roughly between 540 and 650 mμ. The curves for both

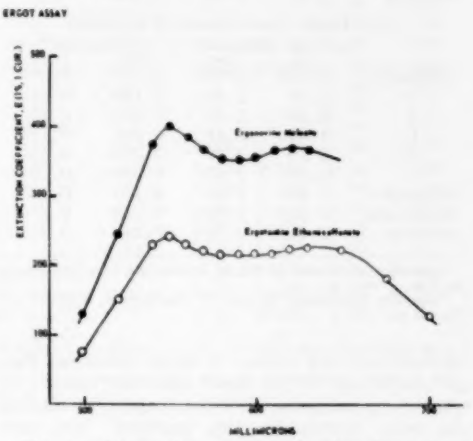

Fig. 1.—Absorption spectra of colors formed by ergonovine maleate and ergotoxine ethanesulfonate as determined with the Beckman Spectrophotometer.

TABLE III.—COMPARISON OF ERGOTOXINE ETHANESULFONATE AND ERGONOVINE MALEATE AS REFERENCE STANDARD COMPOUNDS

Sample[a]	Storage Period	Assay,[b] %
Ergotoxine ethanesulfonate		
Lot B95889W	Before storage	99.7
Lot B95889W	Before storage	99.4
Lot B95889W	1 month	100.0
Lot B12450W	Before storage	95.6
Lot B12450W	2 months	96.6
Lot B12450W	6 months	94.3
Lot B12450W	6 months	96.5
Lot B12450W	7 months	96.9
Lot B12450W	8 months	86.6
Lot B12450W	8 months	87.1
Lot B12450W	8 months	84.0
Lot B12450W	8 months	90.2
Ergonovine maleate, U. S. P.		
Lot 44506	Before storage	100.0
Lot 44506	Before storage	98.6
Lot 44506	1 month	98.6
Lot 44506	1 year	99.5
Lot 44506	1 year 6 months	96.1
Lot 44506	1 year 6 months	98.8
Lot 44506	2 years 9 months	98.1
Lot 44506	2 years 9 months	98.1
Ergonovine maleate, U. S. P., lot proposed as U. S. P. Ref. Std.	Before storage	98.1
Ergonovine maleate, U. S. P., lot proposed as U. S. P. Ref. Std.	Before storage	98.1

[a] The ergotoxine ethanesulfonate was U. S. P. reference standard material.
[b] The highest value in the case of each alkaloidal salt was arbitrarily taken as 100%.

TABLE IV.—FIRST COLLABORATIVE STUDY: TABULATION OF AVERAGE VALUES

(a) Ergot (Two Lots)

Laboratory	Total Alkaloids[a] Lot 58426	Total Alkaloids[a] Lot 58462	Ergonovine[b] Lot 58426	Ergonovine[b] Lot 58462
A	4.06	2.46	0.386	0.362
B	4.49	2.83	0.479	0.497
C	...	...	...	...
D	2.56	2.49	0.318	0.428
E	4.14	2.61	0.352	0.384
Minimum	2.56	2.46	0.318	0.362
Maximum	4.49	2.83	0.479	0.497
Average	3.81	2.60	0.384	0.418

(b) Ergot Fluidextract (Two Lots)

Laboratory	Total Alkaloids[a] Lot 421493	Total Alkaloids[a] Lot 426923	Ergonovine[b] Lot 421493	Ergonovine[b] Lot 426923
A	1.95	2.23	0.189	0.189
B	2.20	2.54	0.245	0.255
C	1.89	2.11	0.207	0.188
D	2.29	2.36	0.215	0.237
E	2.20	2.45	0.206	0.194
Minimum	1.89	2.11	0.189	0.188
Maximum	2.29	2.54	0.245	0.255
Average	2.11	2.34	0.212	0.213

[a] Results calculated as mg. of ergotoxine ethanesulfonate per Gm. or cc.
[b] Results calculated as mg. of ergonovine maleate per Gm. or cc.

alkaloids are very similar in shape indicating that differentiation of the ergot alkaloids cannot be effected by selection of wave lengths in reading the color formed by this reaction. The only significant difference is in intensity, ergonovine forming the more intense color, but it might be pointed out that this does not approach even remotely the difference in biological activity between the two alkaloids. It is evident that the color test by itself can serve only to provide assay values which represent an index of measurement and not a true measure of biological potency.

Further data obtained with the Beckman Spectrophotometer and also with the Lumetron showed for both alkaloids, first, that there was little deviation from Beer's law when the dilution to be tested was in the concentration range, 1–5 mg. per 100 cc., and secondly, that the colors formed were quite stable in the time interval, thirty to sixty minutes following preparation, when read at any wave length in the region of peak absorption. Finally, on repeated trial with the Lumetron, both alkaloids were found to give reproducible extinction coefficients, thus making possible the comparison of all sample readings with predetermined coefficients and obviating the necessity of repeating the determinations of the standard with each assay.

The above findings confirmed that the *p*-dimethylaminobenzaldehyde color reaction is a satisfactory basis for the chemical estimation of the ergot alkaloids. In the present work a wave length of 590 mμ was selected for the color determinations since this approximates the middle of the range of maximum absorption. Any other reading point in the region of maximum absorption would have been acceptable.

Reference Standard Alkaloids.—At the time that this project was undertaken the reference standard most widely accepted in evaluating the alkaloidal potency of ergot and its preparation was ergotoxine ethanesulfonate. In studies carried out by the authors during the course of nearly three years the stability of this compound in the form of the dry

TABLE V.—SECOND COLLABORATIVE STUDY: ASSAY OF ERGOT WITH ADDED PRECAUTIONS

Laboratory		Total Alkaloids[a] Lot 58441	Lot 58462	Lot 58523	Lot 58546	Lot 58563	Ergonovine[b] Lot 58441	Lot 58462	Lot 58523	Lot 58546	Lot 58563
A		...	...	...	3.08	4.15	...	...	...	0.331	0.328
		...	...	...	3.02	4.23	...	...	...	0.327	0.321
	Av.	...	...	...	3.05	4.19	...	...	...	0.329	0.324
B		2.39	2.44	4.46	3.90	4.73	0.324	0.340	0.320	0.392	0.328
		2.44	2.55	4.48	3.90	4.65	0.330	0.342	0.308	0.381	0.306
	Av.	2.41	2.49	4.47	3.90	4.69	0.327	0.341	0.314	0.386	9.317
C		2.35	...	1.95	...	...	0.408	...	0.535	...	...
		2.15	...	1.72	...	...	0.400	...	0.515	...	...
		2.08	...	...	...	...	...	...	...	...	...
		1.98	...	...	...	...	...	...	...	...	...
	Av.	2.14	...	1.83	...	...	0.404	...	0.525	...	...
D		...	...	4.70	3.27	...	...	...	0.266	0.335	...
		...	...	4.90	3.32	...	...	...	0.223	0.342	...
		...	...	4.00	...	...	...	...	0.256	...	...
		...	...	4.10	...	...	...	...	0.245	...	...
		...	...	5.17	...	...	...	...	0.223	...	...
		...	...	6.06	...	...	...	...	0.234	...	...
	Av.	...	...	4.82	3.29	...	...	...	0.241	0.338	...
E		2.14	2.30	...	...	...	0.273	0.263	...	...	...
		2.30	2.31	...	...	...	0.299	0.286	...	...	...
	Av.	2.22	2.30	...	...	...	0.286	0.274	...	...	...
F		...	...	...	2.96	4.2	...	...	...	0.44	0.49
		...	...	...	2.93	4.1	...	...	...	0.42	0.48
	Av.	...	...	...	2.94	4.1	...	...	...	0.43	0.48
Minimum		1.98	2.30	1.72	2.93	4.1	0.273	0.263	0.223	0.327	0.306
Maximum		2.44	2.55	6.06	3.90	4.73	0.408	0.342	0.515	0.44	0.49
Average[c]		2.26	2.39	3.71	3.29	4.34	0.339	0.307	0.360	0.371	0.375

[a] Results calculated as mg. of ergotoxine ethanesulfonate per Gm.
[b] Results calculated as mg. of ergonovine maleate per Gm.
[c] Based on the average value from each laboratory

powder has been compared with that of ergonovine maleate Table III reveals a comparison of assay values determined during this period for two lots of U. S. P. Ergotoxine Ethanesulfonate Reference Standard and two lots of Ergonovine Maleate U. S. P. taking the highest value for each alkaloidal salt as 100%. A spread of 16% for ergotoxine ethanesulfonate compared with a spread of only 4% for ergonovine maleate. Some decomposition was apparent in the case of lot B12450W of ergotoxine ethanesulfonate, which showed a drop in assay values of about 10% in eight months' time. Greater stability was demonstrated by ergonovine maleate notwithstanding the fact that the ergotoxine ethanesulfonate was sealed in small vials under an atmosphere of nitrogen, while intermittent exposure to the air was permitted in the case of the ergonovine maleate. Both compounds were stored in the dark at room temperature throughout the course of these studies. It was concluded that ergonovine maleate as the dry powder is stable for periods of longer than two years when stored in the dark at room temperature in a vessel containing air and that it is to be preferred to ergotoxine ethanesulfonate for use as a reference standard in a chemical assay method.

TABLE VI.—SECOND COLLABORATIVE STUDY: ASSAY OF ERGOT FLUIDEXTRACT WITH ADDED PRECAUTIONS

Laboratory		Total Alkaloids[a] Lot 434400	Total Alkaloids[a] Lot 448142	Ergonovine[b] Lot 434400	Ergonovine[b] Lot 448142
A		2.70	2.99	0.305	0.251
		2.70	3.02	0.310	0.255
		2.68	2.99	0.287	0.252
		2.67	3.00	0.299	0.254
		2.62	...	0.277	...
		2.65	...	0.286	...
	Av.	2.67	3.00	0.294	0.253
B		3.01	3.51	0.270	0.276
		3.07	3.49	0.292	0.272
		3.23	...	0.288	...
		3.21	...	0.296	...
	Av.	3.13	3.50	0.286	0.274
C		2.35	...	0.43	...
		2.42	...	0.56	...
		2.42	...	0.228	...
		2.45	...	0.258	...
	Av.	2.41	...	0.369	...
D		2.84	3.11	0.182	0.181
		2.84	3.09	0.177	0.184
		2.88	...	0.181	...
		2.94	...	0.174	...
	Av.	2.87	3.10	0.178	0.182
E		2.49	2.67	0.214	0.170
		2.48	2.69	0.226	0.180
		2.34	2.70	0.214	0.180
		2.43	2.76	0.198	0.190
	Av.	2.43	2.70	0.213	0.180
F		2.6	2.8	0.36	0.33
		2.6	2.8	0.35	0.32
	Av.	2.6	2.8	0.35	0.32
Minimum		2.34	2.67	0.174	0.170
Maximum		3.23	3.51	0.56	0.33
Average[c]		2.68	3.02	0.282	0.243

[a] Results calculated as mg. of ergotoxine ethanesulfonate per cc.
[b] Results calculated as mg. of ergonovine maleate per cc.
[c] Based on the average value for each laboratory.

TABLE VII.—THIRD COLLABORATIVE STUDY: UNIFORM REFERENCE STANDARD

Laboratory		Ergot (1 Lot) Total Alkaloids[a]	Ergot (1 Lot) Ergonovine[b]	Ergot Fluidextract (1 Lot) Total Alkaloids[a]	Ergot Fluidextract (1 Lot) Ergonovine[b]
A		4.59	0.449	2.01	0.215
		4.61	0.455	1.98	0.219
	Av.	4.60	0.452	1.99	0.217
B		5.01	0.478	2.20	0.246
		4.91	0.487	2.23	0.249
	Av.	4.96	0.482	2.21	0.247
D		...	...	2.04	0.095
		...	...	2.00	0.099
	Av.	...	...	2.02	0.097
E		8.53	0.445	1.88	0.183
		...	...	1.89	0.184
		...	...	1.85	0.184
		...	...	1.88	0.183
	Av.	8.53	0.445	1.88	0.183
F		3.55	0.431	1.04	0.092
		3.55	0.431	1.04	0.092
		3.55	0.431	1.04	0.092
		3.55	0.431	1.04	0.092
	Av.	3.55	0.431	1.04	0.092
Minimum		3.55	0.431	1.04	0.092
Maximum		8.53	0.487	2.23	0.249
Average[c]		5.41	0.452	1.83	0.167

[a] Results calculated as mg. of ergotoxine ethanesulfonate per Gm. or cc.
[b] Results calculated as mg. of ergonovine maleate per Gm. or cc.
[c] Based on the average value for each laboratory.

In order to make possible the calculation of total alkaloids in terms of ergotoxine ethanesulfonate or ergotoxine alkaloid, while using ergonovine maleate as the reference standard, it was necessary to develop a suitable conversion factor based on actual performance of the pure alkaloidal salts in the color reaction. The factor obtained by the authors, representing an average of many determinations, was 1.610 for converting from ergonovine maleate to ergotoxine ethanesulfonate, or 1.370 calculated in terms of ergotoxine alkaloid itself.[3]

Collaborative Studies.—The assay methods for ergot and ergot fluidextract developed by the authors were first submitted for trial to the Subcommittee on the Chemical Assay of Ergot before addition of further precautionary instructions, and the results of this first collaborative study, which have already been published, are reproduced in Table IV. A second study was undertaken after taking most of the previously discussed precautions into consideration, and the results, not covered by previous publication, appear in Tables V and VI. Finally, a third collaborative study was made without significant change of the methods, but with a slightly different approach in that a reference standard was supplied with the samples (Table VII). This reference standard was a uniform sample of Ergonovine Maleate, U. S. P., which was dispensed

[3] As a result of the recommendations of the Subcommittee on the Chemical Assay of Ergot, Ergonovine Maleate Reference Standard is now included in the "United States Pharmacopeia," Fourteenth Revision, and Ergotoxine Ethanesulfonate Reference Standard has been omitted. The factor, 1.370, for conversion from ergonovine maleate to ergotoxine alkaloid has been accepted in "The National Formulary," Ninth Edition, where it appears in the *Assay for total alkaloids* under *Ergot*.

in the form of the dry powder. (U. S. P. Ergonovine Maleate Reference Standard was not available at the time this study was undertaken.) In connection with this last study the reproducibility of the color test by itself was also investigated by the collaborators, and these results are reported in terms of the extinction coefficients (Table VIII).

TABLE VIII.—COLLABORATIVE STUDY OF REPRODUCIBILITY OF COLOR TEST AS APPLIED TO UNIFORM REFERENCE STANDARD (ERGONOVINE MALEATE)

Laboratory		Extinction Coefficient, E (1%, 1 Cm.)[a] Ergonovine Maleate, 2 Mg./100 Cc.[b]	Ergonovine Maleate, 4 Mg./100 Cc.[b]
A		393	387
		396	390
	Av.	394	388
B		346	347
		341	346
	Av.	343	346
D		362	362
		362	362
		362	...
	Av.	362	362
E		343	349
		336	340
		345	351
		333	340
		330	336
		333	339
	Av.	337	342
F		375	374
		375	374
		375	374
		375	374
		374	373
		374	373
		374	373
		374	373
	Av.	375	374
Minimum		330	336
Maximum		396	390
Average[c]		362	362

[a] The values given are based on the approximate concentration of the drug in the final colored solutions, without taking into account the slight volume contraction on adding the reagent to the aqueous dilutions.

[b] The concentrations here refer to the initial aqueous dilutions.

[c] The final over-all averages are based on the average value for each laboratory.

The values given are absolute values, while the usual practice in colorimetry is to calculate assay values on a relative basis, thereby eliminating for the most part any instrument errors. The results indicated that failure of the ergot color test was not responsible to any appreciable extent for deviations in the collaborative assay results.

Examination of the collaborative figures revealed that each analyst generally reproduced his own values quite satisfactorily when assaying either ergot or the fluidextract, while at the same time considerable deviations were seen to be common on comparing the work of different laboratories. Laboratories *A* and *B* in Tables IV, V, VI, and VII, which were the same laboratories in each case, represented the two having the most experience

TABLE IX.—ANALYSIS OF FIGURES IN TABLES IV, V, VI, AND VII: COMPARISON OF PER CENT DEVIATION FROM THE MEAN

Collaborative Study	Average Per Cent Deviation from Mean[a] —Ergot Assay— Total Alkaloids	Ergonovine	—Fluidextract Assay— Total Alkaloids	Ergonovine
	(*a*) All Laboratories			
1	10.6	11.7	6.4	9.6
2	11.7	16.7	7.6	20.1
3	28.8	3.2	17.2	34.8
	(*b*) Laboratories *A* and *B* Alone			
1	6.0	13.2	6.3	13.9
2	8.9	1.2	7.8	2.7
3	3.8	3.2	5.2	6.5

[a] Based on the average assays reported by each collaborating laboratory.

with the methods involved, and these demonstrated the greatest ability to cross-check. An analysis of all the collaborative figures has been condensed in a single table (Table IX) by presenting the average deviations from the mean for each of the three collaborative studies. A similar comparison has also been drawn for laboratories *A* and *B* alone. This analysis reveals a slight improvement in the uniformity of results submitted by laboratories *A* and *B* during the course of the three studies, but unfortunately some of the results turned in by certain of the other laboratories in collaborative study number 3 were unaccountably erratic even though several possible sources of error had been removed which might have interfered in the case of the first series of collaborative assays. It is believed that practice in the use of assay methods such as these plays an important role in obtaining the most precise results.

SUMMARY

1. The complete work involved in development of new assay methods for ergot and ergot fluidextract, under the sponsorship of the Subcommittee on the Chemical Assay of Ergot, is covered in a single report. The earlier part of the work, which has previously been published (3), is reviewed. Also presented are certain heretofore unpublished results concerning the colorimetric step, reference standard alkaloids, and collaborative studies.

2. While no method for ergot fluidextract has been admitted to the N. F., the assay procedure developed for ergot has been judged satisfactory on the basis of this study and is now official in the N. F. IX.

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A Study of the Potentiation of Pentobarbital Anesthesia by Glucose and Its Metabolites*

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It was shown that the injection of concentrated solutions of glucose into rats immediately after recovery from pentobarbital anesthesia caused a return to anesthesia. The simultaneous administration of glucose and pentobarbital resulted in a longer period of anesthesia than did the injection of pentobarbital alone. The effect of various glucose metabolites upon blood glucose levels and upon blood *p*H in rats was studied. A comparison of the levels of pentobarbital in the liver, brain, and blood of rats following administration of pentobarbital alone, and pentobarbital and glucose, was made. Also investigated was the influence of high blood glucose levels upon the induction time of anesthesia and upon sleeping time produced by pentobarbital. A possible mechanism of glucose potentiation of pentobarbital anesthesia is suggested.

In 1949 Lamson and his co-workers (1) made the observations which first aroused interest in this investigation. They were studying shock following hemorrhage in dogs under pentobarbital anesthesia and found that the intravenous injection of glucose into dogs immediately after recovery from anesthesia resulted in a prompt return to anesthesia. This observation, substantiated by subsequent experimentation, led them to the belief that glucose, a large number of its intermediary metabolites, and various other substances are able to potentiate pentobarbital anesthesia. Similar results were reported by us (2) in experiments on rabbits. It was found that glucose administration resulted in a higher blood level of pentobarbital than was obtained if pentobarbital were given without glucose. Preliminary studies on minced liver tissue indicated an interrelationship between the rate of glycogen polymerization and the rate of pentobarbital uptake by the liver. Ratcliff, *et al.* (3), had shown a similar relationship in studies on Pentothal.

In the belief that potentiation of pentobarbital by glucose would have pharmaceutical and therapeutic applications, it was decided that further study of the problem was warranted.

EXPERIMENTAL

Since it was desired to use rats in part of the experimental procedures, it was necessary to establish the presence of the "glucose effect" in these animals. Only Wister strain, albino rats were used. Their ages ranged from three to five months. The animals were housed in a room in which a temperature of 76 to 78° F. was maintained. Their diet consisted of Purina Dog Chow and water, *ad libitum*. Prior to use in experimentation, the rats were deprived of food, but not of water, for a period of eighteen to twenty-four hours.

In all cases, return of the righting reflex was used as a criterion of recovery from barbiturate depression.

The presence of the glucose effect in rats was established in two ways. In the first of these, rats were anesthetized by the intraperitoneal injection of 2.75 mg. of pentobarbital per 100 Gm. of rat. Upon recovery from anesthesia, 2 cc. of a 50% solution of glucose were administered intraperitoneally. This injection caused a return to anesthesia in 10 of 11 female rats, and in all of 5 male rats. The recovery times following glucose injection averaged 22.1 ± 3.5 minutes in females, and 16.2 ± 3.7 minutes in males.

The second procedure to establish the ability of glucose to potentiate pentobarbital in rats was carried out as follows: rats were anesthetized by intraperitoneal injection of pentobarbital and the resulting recovery times were recorded. After a delay of a week, the same rats were given an identical dose of pentobarbital, but this time the drug was accompanied by the injection of 2 cc. of 50% glucose. The recovery times were again recorded. It was found that, in twenty females, the mean recovery times following pentobarbital was 45.2 ± 4.8 minutes; following pentobarbital and glucose it was 77.7 ± 4.0 minutes. In ten males pentobarbital injection resulted in a mean recovery time of 26.0 ± 3.5 minutes, whereas pentobarbital and glucose combined caused an anesthesia of 48.2 ± 3.4 minutes' duration. On the basis of these results, rats were deemed to be suitable animals for use in further studies. It had been established earlier that rabbits are similarly affected by glucose (2).

In addition to potentiating pentobarbital, the injection of a concentrated solution of glucose obviously would cause an increase in the level of blood sugar. Various glucose metabolites have been reported to potentiate barbiturate anesthetics (4), so it seemed logical to determine the effect of these metabolites on the level of blood glucose, if any. This study was made on rabbits. By making a

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small incision in the marginal ear veins of rabbits, 0.1 cc. of blood was obtained, and assayed for glucose by the Folin-Malmros technique (5). Then 3 cc. of 20% sodium lactate were injected intraperitoneally. At periods of ten, twenty-five, forty, and sixty minutes after this injection, 0.1 cc. blood samples were withdrawn and their glucose content determined. Similar determinations were made to find the effect of sodium citrate, sodium acetate, sodium succinate, sodium pyruvate, sodium chloride (as a control), and finally of glucose itself (2 cc. of a 50% solution). The level of blood sugar in each sample from each rabbit was then computed as a per cent of the normal for that animal at that time, normal being taken as 100%. The maximum change and the mean change over the sixty minutes, produced by each substance, are shown in Table I.

TABLE I.—THE EFFECT OF SOME GLUCOSE METABOLITES ON BLOOD SUGAR LEVELS IN RABBITS[a]

Metabolite	Maximum Change, %	Mean Change, %
Glucose	146.2 ± 6.5	137.2 ± 4.2
Sodium lactate	116.5 ± 4.0	112.7 ± 2.3
Sodium citrate	114.5 ± 4.3	112.2 ± 3.1
Sodium acetate	116.5 ± 3.3	111.6 ± 2.1
Sodium succinate	108.0 ± 4.0	105.3 ± 3.7
Sodium pyruvate	114.2 ± 4.3	111.4 ± 2.8
Sodium chloride	109.3 ± 5.2	100.1 ± 4.2

[a] Each value is the mean response of not less than ten rabbits. In all cases, normal was taken as 100 per cent.

Because it had been ascertained earlier that the injection of 2 cc. of 50% glucose caused a return to anesthesia in only 50% of the animals injected (2), it appeared doubtful that the metabolites could potentiate through their effect on blood sugar, since their effect was much less than that of glucose itself.

Subsequent experiments in rats were carried out with the various metabolites in an attempt to cause a return to anesthesia. Graded doses of sodium lactate, sodium citrate, sodium acetate, sodium succinate, and sodium pyruvate were all tried. Responses in rats ranged from none, through various degrees of depression, to death. None of the animals, however, was returned to anesthesia. The symptoms in those animals which showed a response included anoxia, cyanosis, tremors, convulsions, and, in some cases, death. Those animals that recovered urinated excessively for some hours after recovery. These are the typical symptoms of alkalosis. Attempts to buffer the solutions so as to prevent the development of these symptoms on injection were unsuccessful. Lamson and his group had reported potentiation could be produced by the glucose metabolites. Our inability to show similar results was probably due to the use of different criteria in deciding whether the animals were anesthetized, in that we did not consider the animals to be returned to anesthesia if they responded to stimuli.

The injection of neither glucose nor sodium lactate caused any appreciable change in the *p*H of the blood. This finding, however, does not rule out the probability that alkalosis had followed the administration of the sodium salts of the glucose metabolites. It has been demonstrated that alkalosis is not necessarily associated with a change in the *p*H of the blood (6).

Even though potentiation of pentobarbital was not shown by the sodium salts of the glucose metabolites, glucose itself exhibited very definite potentiating ability, hence subsequent experimentation was confined to a study of the action of glucose.

In the first phase of this study, we investigated the effect of glucose upon the levels of pentobarbital in the liver, brain, and blood of female rats three to five months old. By intraperitoneal injection, rats were given 3 mg. of pentobarbital per 100 Gm. At periods of ten, thirty, and sixty minutes after injection, and at recovery from anesthesia, the animals were killed by a blow on the head. The liver and brain were removed immediately, and sliced. A portion of the liver was assayed for glycogen content by a method described by Koch and Hanke (7). The remainder of the liver and the brain were homogenized separately, and the pentobarbital content of each was determined spectrophotometrically (8). Blood samples were assayed similarly for both pentobarbital and glucose. In Tables II, III, IV, and V, each value represents an average of 12 determinations.

TABLE II.—LEVELS OF PENTOBARBITAL IN THE LIVER, BLOOD, AND BRAIN FOLLOWING PENTOBARBITAL ALONE

	Liver, Mcg./Gm.	Blood, Mg./100 Cc.	Brain, Mcg./Gm.
Ten minutes	284 ± 15.5	1.54 ± 0.27	199 ± 16.9
Thirty minutes	154 ± 12.7	1.18 ± 0.28	208 ± 17.0
Sixty minutes	161 ± 16.9	1.01 ± 0.24	177 ± 9.6
At recovery	79 ± 12.6	0.29 ± 0.08	53 ± 10.6

TABLE III.—LEVELS OF PENTOBARBITAL IN THE LIVER, BLOOD, AND BRAIN FOLLOWING PENTOBARBITAL AND GLUCOSE

	Liver, Mcg./Gm.	Blood, Mg./100 Cc.	Brain Mcg./Gm.
Ten minutes	187 ± 7.3	4.61 ± 0.42	200 ± 13.2
Thirty minutes	112 ± 19.3	3.90 ± 0.87	207 ± 33.8
Sixty minutes	92 ± 9.0	3.55 ± 0.38	180 ± 17.2
At recovery	90 ± 2.7	0.92 ± 0.19	98 ± 10.0

TABLE IV.—LEVELS OF BLOOD GLUCOSE AND LIVER GLYCOGEN FOLLOWING PENTOBARBITAL ALONE

	Blood Glucose, Mg./100 Cc.	Liver Glycogen, Mg./Gm.
Normal	79 ± 2.9	2.15 ± 0.10
Ten minutes	90 ± 2.9	1.79 ± 0.12
Thirty minutes	97 ± 2.2	2.32 ± 0.36
Sixty minutes	79 ± 1.5	2.63 ± 0.24
At recovery	81 ± 1.9	5.80 ± 0.23

Following this, identical procedures were carried out after the injection of 3 mg. of pentobarbital plus 1 cc. of 50% glucose per 100 Gm. of rat.

From Tables II and IV, it is readily seen that the injection of glucose along with pentobarbital results in an increased rate of build-up of liver glycogen, accompanied by a decrease in accumulation of pentobarbital in the liver. In an attempt to verify these findings, a further study of this interrelationship was made. Livers from freshly killed animals were homogenized. To 30 cc. volumes of homogenate were added 3 cc. of a solution containing 60 mg. pentobarbital per cc., and either 27 cc. of 0.9% sodium chloride, or 27 cc. of 20% glucose. The mixtures were incubated at 37° for fifteen minutes. They were then filtered, and the filtrate was assayed for glucose and for pentobarbital. From these results it was possible to compute the amounts of glucose and of pentobarbital taken up by the liver. In the absence of glucose, pentobarbital was removed at the rate of 1.53 ± 0.39 mg. per Gm. of liver. However, when glucose was added, the liver removed from solution 122.7 mg. glucose per Gm. of liver, but only 0.78 ± 0.24 mg. pentobarbital per Gm. of liver. A significant difference in the rate liver removes pentobarbital from the solutions rich in glucose and those deficient in glucose is indicated by $t = 2.744$.

TABLE V.—LEVELS OF BLOOD GLUCOSE AND LIVER GLYCOGEN FOLLOWING PENTOBARBITAL AND GLUCOSE

	Blood Glucose, Mg./100 Cc.	Liver Glycogen, Mg./Gm.
Ten minutes	388 ± 27.7	3.83 ± 0.14
Thirty minutes	393 ± 23.2	5.22 ± 0.51
Sixty minutes	380 ± 16.9	6.04 ± 0.19
At recovery	232 ± 8.4	9.88 ± 0.14

If incubation of these solutions was continued for forty-five minutes, and the filtrates then injected into rats, it was found that the induction times and recovery times were as follows: the solution incubated only with pentobarbital produced anesthesia in female rats in 5.8 ± 0.53 minutes, and in males in 5.1 ± 0.32 minutes. Females were anesthetized 121 ± 9.7 minutes, males 47 ± 5.0 minutes. The solution incubated with pentobarbital and glucose induced anesthesia in females in 6.1 ± 0.55 minutes, and in males in 5.3 ± 0.61 minutes. The recovery times were 127 ± 13.9 minutes in females; 54 ± 5.8 minutes in males. Statistical evaluation showed the difference to be insignificant, even though in all cases the pentobarbital solution incubated with liver and glucose appeared to be more potent.

Administration of glucose ten minutes before giving pentobarbital, thereby producing high blood sugar levels, had no effect on the induction times of anesthesia, even though the blood level of pentobarbital is higher at the onset of anesthesia if glucose is given than it is when glucose is not given.

DISCUSSION

There have been a number of mechanisms suggested as possible explanations of the phenomena involved in glucose potentiation of pentobarbital anesthesia. On the basis of the experimental results outlined herein, it seems most likely that the site of the potentiation is the liver. In this organ there is an inverse relationship between the formation of glycogen and the removal of pentobarbital from the blood. That observation immediately suggests the possibility that there is a common enzyme system involved in the two processes. A report by Gould and Shideman (9) makes this appear likely. It states that high-energy phosphate-bound compounds are required by the liver in order to detoxify thiopental. In addition, a number of enzyme inhibitors, whose action is to prevent phosphorylation, prevent thiopental detoxification. They also prevent glycogen polymerization. From this it can be assumed that phosphorylation is involved in both glycogen formation and barbiturate detoxification. In the presence of excessively high glucose concentrations, it seems logical that some of the compounds which detoxify pentobarbital are being utilized in glycogenesis. Hence, excess glucose would delay destruction of the drug, resulting in prolonged action.

The depressant action of barbiturates upon the central nervous system is brought about by inhibition of glucose utilization of that system. Following the administration of highly concentrated solutions of glucose, nervous tissue metabolism would obviously be increased, necessitating the presence of greater amounts of pentobarbital to inhibit this increased metabolism and cause anesthesia. The fact that blood levels of pentobarbital are considerably higher at onset of anesthesia if glucose is given with the drug substantiates this hypothesis.

In conclusion, there seem to be two factors involved in the simultaneous administration of pentobarbital and glucose. The added glucose enables the central nervous system to resist depression, but the resulting liver glycogenesis causes a slower detoxification of pentobarbital. The overall effect is a prolongation of pentobarbital depression.

SUMMARY

1. Glucose prolongs the recovery time from anesthesia induced by pentobarbital in rats.

2. Rats, upon recovery from pentobarbital anesthesia, are returned to anesthesia by the injection of concentrated solutions of glucose.

3. Sodium citrate, sodium lactate, sodium acetate, sodium succinate, and sodium pyruvate

produce the symptoms of alkalosis but do not return rats to anesthesia.

4. The levels of pentobarbital in the blood and brain become higher if glucose is administered with the barbiturate than if pentobarbital is given alone.

5. The liver does not remove pentobarbital from the blood as efficiently in the presence of excess glucose as it does in the absence of glucose.

6. The female rat is more susceptible to barbiturate depression than is the male.

7. Glucose potentiates pentobarbital anesthesia in both the rat and the rabbit.

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The *in vitro* Evaluation of Conspergents on Selected Enteric Coatings*

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A study was made of conspergents which may be suitable for use with enteric coating solutions. Brenner's modified method and the British Pharmacopoeial method were used. It was found that disintegration rates varied with the methods employed and the number of applications necessary for an enteric coating varied with the substances used.

THE IMPORTANCE of conspergents with coating materials was recognized as early as 1849 when Jozeau (1) used specially prepared ammoniacal powdered casein with a mucilage of powdered casein in water. Proctor (2), in 1862, used powdered sugar, tragacanth, talc, lycopodium, magnesia, and pumice with various combinations of coating solutions. Magnes-Lahens (3), in 1874, used acacia powder while talc was used in the pearl coating process (4).

The importance of the selection of a conspergent with suitable properties may be further exemplified by the experiments conducted by Bukey and Klemme (5) in 1939. Talc and magnesium stearate were used with a solution of mastic in methyl propyl ketone. The efficiency of the tablets coated with mastic and talc was 42.5 per cent as compared to an efficiency of 62.5 per cent for those coated with mastic and magnesium stearate. Thompson and Lee (6) employed magnesium stearate as a conspergent in their experiments in 1945.

It is evident that the selection of a conspergent is of great importance in the field of enteric coatings, for the purposes of improving the appearance and bringing about selective disintegration in the desired portion of the digestive tract. Water-soluble substances and powders which react in the acid medium of the stomach would be unsatisfactory for use because of the tendency to cause disintegration before the tablet or pill reaches the intestine. For this reason, an inert powder or one which has a tendency to dissolve or become emulsified in the intestinal juice should be selected. Since a study of the physiology of the digestive tract indicates that the intestinal contents vary from distinctly acid to slightly alkaline (7), a conspergent which is effective in alkaline and acid media should be chosen.

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DEFINITION AND IMPORTANCE OF ENTERIC COATINGS

By definition, an enteric coating is one that resists the action of the stomach fluids and disintegrates in the intestine (8).

The importance of enteric coatings was shown in a survey conducted in 1931 (9) in which 3.3% of the prescriptions obtained from 64 pharmacies were for enteric coated products. Mordell (10), in 1949, conducted a prescription study including 13,125 preparations. Of that number 256 were for sodium salicylate of which 91 or 35.2% were for the enteric coated form.

Uses of Enteric Coatings.—A further illustration of the importance of enteric coatings may be noted in the uses which may be ascribed to them. They are: (*a*) to prevent gastric digestion or decomposition of the drug, such as glandular products and pancreatin; (*b*) to prevent nausea and vomiting caused by the drug, for example, emetine, atabrine, diethylstilbestrol, and sulfonamides; (*c*) to prevent dilution of the drug before reaching the intestine, for example, intestinal antiseptics and anthelmintics; (*d*) to prevent hindrance of gastric digestion by the drug, such as alkaline medicaments; (*e*) to give delayed action of the drug for such substances as the sulfonamides, aspirin, and the barbiturates; and (*f*) to provide a simple means of administering drugs for use in the intestine (6).

A review of the products marketed by pharmaceutical manufacturers in enteric form indicates further the importance of enteric coatings.

Physiological Factors Pertinent to Enteric Coatings.—The normal stomach after a test meal has a *p*H of 1.67 and after combination of the acid with protein, the *p*H is 3.0 (11). The temperature of the stomach varies from 97.5° to 102.2° F. (7), and the emptying time varies from four to five hours after an average meal (12). The emptying time is shortest with a carbohydrate diet (13) and tablets leave an empty stomach more quickly than a full stomach (14). Goorley and Lee (15) found that enteric medication left the stomach in a more uniform time if given two hours before meals.

The enzymes in the stomach include pepsin, rennin, gastric lipase, and a small amount of tryspin. After the material leaves the stomach, it comes into intimate contact with the bile and pancreatic juice. The enzymatic constituents of the pancreatic juice include trypsin, chymotrypsin, various carboxypeptidases, polynucleotidase, amylase, and lipase (16). Bile contains salts, sodium glycocholate and sodium taurocholate, pigments bilirubin and biliverdin, cholesterol, and lecithin (17). Intestinal juice contains peptidases, carbohydrases, enterokinase, and phosphatase (16).

Best and Taylor (17) point out that though the chemical aspect of digestion is now held to be most important, the mechanical factors must not be overlooked.

It may be noted that the small intestine may vary from slightly alkaline to distinctly acid and that the reaction found is a result of an equilibrium of the acid chyme and the alkaline juices of the duodenum (18). Bodansky (12) states that the lower duodenum and the jejunum are best adapted for absorption. Howell (11) has shown on dogs that the *p*H of the duodenum during digestion varied from 3.8 to 6.6, that the jejunum was slightly acid in reaction, but the ileum did not drop below a *p*H of 7. Wruble (7) stated that the temperature of the intestine varied from 98° to 100.1° F. and that the peristaltic motion began soon after the ingestion of a meal.

Factors Influencing the Disintegration of Enteric Coatings.—Disintegration of enteric coatings is usually dependent on one or more of the following factors.

(*a*) An alkaline medium. (This class is exemplified by the synthetic polymerization products of definite acid number and ammoniacal shellac.)

(*b*) Enzymatic hydrolysis in the intestine. (The use of oils and fats which are saponifiable in the intestine are examples of this class.)

(*c*) Other chemical reactions such as the neutralization reaction. (This means is seldom used.)

(*d*) Timed disintegration made possible by the use of hygroscopic substances which in the presence of moisture swell and cause the coating to fall apart.

(*e*) Disintegration by the use of agents like bile and cholesterol (6).

Summary of a Good Enteric Coating.—After consideration of the physiology of the digestive tract, it appears that an efficient enteric coating is one that is stable in the stomach for five hours, and disintegrates in the intestine, preferably within one hour (6). In addition to the satisfactory disintegration, a coating should have a good appearance, be adaptable for use commercially, be easy to apply, economical, and remain stable under normal storage conditions.

EXPERIMENTAL

Coating Machines.—A small coating machine (Fig. 1) similar to that described by Thompson and Lee (6) was designed to coat from 200 to 300 tablets. The average speed of the coating bowl was 33 revolutions per minute.

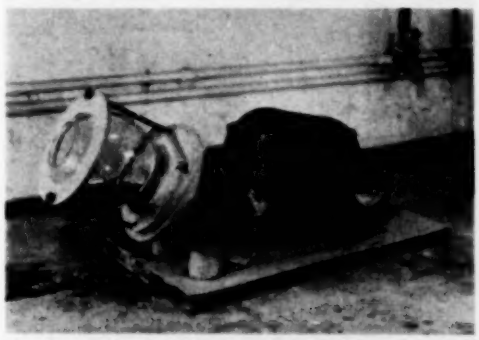

Fig. 1.—Tablet coating machine.

Later a machine capable of rotating four bowls of the same size was made (Fig. 2). A commercial coating machine with an average pan speed of 36 revolutions per minute supported a masonite disk 36 cm. in diameter. Five wheels 13.5 cm. in diameter were attached to the disk. Each wheel was made of two disks 13.5 cm. in diameter, with two

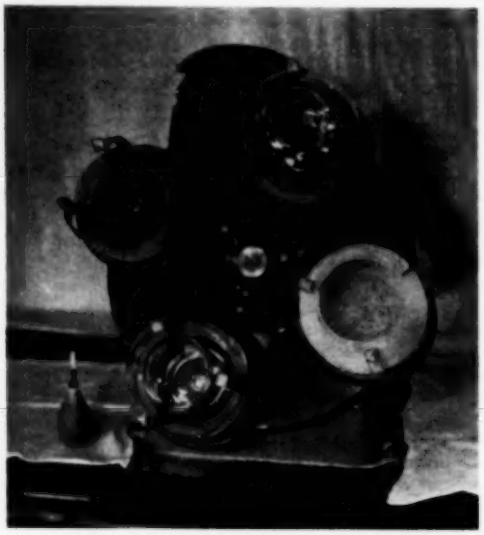

Fig. 2.—Multiple coating pan machine.

disks between of approximately 12.5 cm. diameter, which were bolted together. The center wheel was attached to the shaft of the coating machine by means of a brass collar and a set screw. A belt of sponge rubber 1 cm. thick and 1.5 cm. wide fitted into the depression between the two outer disks of each wheel. Each wheel could be tightened against the center wheel or removed from contact by a slot in the large disk. Three brass supports were attached to the face of four of the wheels. The average speed of the bowls was 36 revolutions per minute.

Fig. 3.—Disintegration apparatus for Brenner modification.

Devices and Methods of Applying Solutions.—Three methods were used to apply the enteric coating solutions. A medicine dropper was used for the application of the stearic acid, mutton tallow, balsam tolu, sodium taurocholate to the pills, a 5-ml. pipette was used to apply the shellac and cetyl alcohol, sandarac, tolu coatings to the pills and subcoated tablets, and an atomizer was used to apply the stearic acid, mutton tallow coatings to the subcoated tablets.

Devices and Methods of Applying Powders.—An infant syringe was used to apply the conspergents.

Drying Apparatus.—An electric drier was used with the single bowl coating machine. The drier for the multiple pan coating machine consisted of a flexible metal tube fitted on an electric drier. The temperature of the air was approximately 30°.

Disintegration Apparatus.—Two methods for the testing of disintegration were employed each requiring an apparatus which was designed.

The first method for determining disintegration (Fig. 3) was similar to that used by Brenner (19) in 1943. Ten holes were made in 11-cm. sharkskin filter paper, the perforated paper tied over a 50-ml. beaker, and placed in a 150-ml. beaker containing the artificial digestive solution. The surface of the liquid was approximately 1 cm. above the tablet. A rack in the constant temperature bath supported the beakers.

The apparatus employed for the second method of testing disintegration (Fig. 4) was similar to that recommended by the *British Pharmacopoeia* (20). A plyboard frame, 21.7 cm. wide, 8 cm. long, and 2.5 cm. thick was bolted on a metal axle with a 4-in. pulley, and mounted on a steel base. Two sets of rubber holders were attached to the plyboard frame. One held eight test tubes 15 cm. in length and 1.5 cm. inside diameter; the other, four test tubes 14.7 cm. in length and 2.5 cm. inside diameter. The tubes were closed with rubber finger cots of suitable size and the apparatus immersed in a constant temperature bath (Fig. 5) at 37.5°.

SELECTION AND EXPLANATION OF CONSPERGENTS

A study was made of the conspergents available which may be suitable for use with enteric coating solutions. The selection was based on appearance, solubility, availability, and mobility. Two pow-

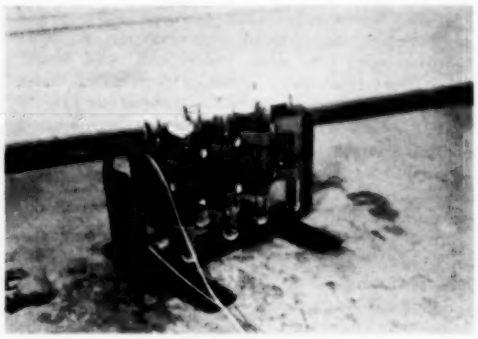

Fig. 4.—Test tube support for British Pharmacopoeial modification.

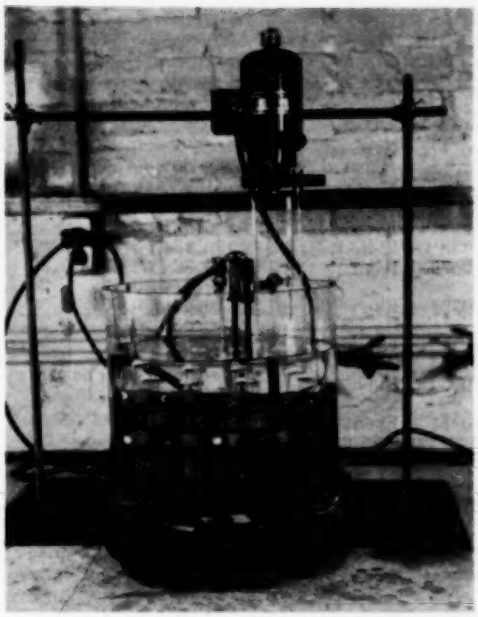

Fig. 5.—Disintegration apparatus for British Pharmacopoeial modification.

ders, talc and subcoating dusting powder, were employed as recommended by Rowell (21) for use with two enteric formulas. The subcoating powder consisted of 48 Gm. precipitated calcium carbonate, 25 Gm. of corn dextrin,[1] 2 Gm. of talc, and 25 Gm. of powdered sugar. It was recommended for use with cetyl alcohol, sandarac, tolu coating, while talc was used with the shellac coating. The other conspergents used were: kaolin; bentonite; Magnesol (a commercial form of magnesium trisilicate); magnesium trisilicate; tertiary magnesium phosphate; magnesium oxide; magnesium stearate; calcium stearate; aluminum stearate; aluminum palmitate; Dry-Flo starch (a non-wettable starch manufactured by National Starch Products); ethyl *p*-aminobenzoate; methylparaben; and *n*-propylparaben.

The powders were commercial products with the exception of the calcium stearate which was made in the laboratory.

Consideration of the physical properties of the conspergents did not lead to conclusive results in regard to selection of a powder for use with enteric coating formulas. The satisfactory conspergents varied in chemical composition, specific gravity, and melting points. In the determination of particle size, the satisfactory powders were over 50% finer than 200 mesh. Excellent mobility and insolubility in alcohol characterized the satisfactory conspergents to some extent. Magnesium stearate and calcium stearate appeared to have a selective behavior when used.

[1] Tapioca dextrin was not available.

TABLE I.—STEARIC ACID, MUTTON TALLOW COATINGS

Conspergent	Amt. Soln., Ml./ Coat	Amt. Powd., Gm./ Coat	Ratio, Gm./ Ml.	Thickness, Mm.	Description	Disintegration: Method[a]	Disintegration: Acid Pepsin Soln., Hr.	Disintegration: Alkaline Pancreatin Soln., Hr.	Disintegration: Acid Pancreatin Soln., Hr.
Talc	0.40	0.568	1.420	0.1120	Even	1	7.80	0.60	4.5
						2	3.67	0.17	3.68
Kaolin	0.80	0.100	0.125	0.1170	Uneven	1	3.25	0.22	0.50
						2	5.72	0.18	0.68
Bentonite	1.75	1.100	0.629	0.2475	Rough	1	0.12	0.08	0.08
						2	0.13	0.08	0.08
Magnesol	0.42	0.345	0.822	0.3805	Rough	1	2.23	0.08	1.35
						2	0.42	0.15	0.37
Magnesium trisilicate	0.52	0.333	0.640	0.0890	Uneven	1	0.28	0.28	0.08
						2	0.20	0.10	0.07
Tertiary magnesium phosphate	1.10	0.200	0.182	0.1587	Uneven	1	1.05	0.23	1.45
						2	0.88	0.13	0.43
Magnesium oxide	1.20	0.110	0.092	0.1188	Uneven	1	0.63	5.50	3.13
						2	1.48	1.80	1.95
Magnesium stearate	0.29	0.265	0.914	0.1320	Even	1	8[b]	0.15	0.43
						2	4.88	0.22	0.33
Calcium stearate	0.21	0.236	1.124	0.2130	Even	1	8[b]	1.47	1.43
						2	5.75	0.33	0.47
Aluminum stearate	0.35	0.478	1.366	[c]	Rough	1	0.08	0.05	0.05
						2	0.17	0.03	0.02
Aluminum palmitate	0.37	0.202	0.546	0.1190	Uneven	1	7.38	0.72	2.15
						2	2.38	0.45	1.68
Dry-Flo starch	0.26	0.584	2.246	0.2155	Uneven	1	0.15	0.05	0.15
						2	0.03	0.03	0.03
Ethyl *p*-aminobenzoate	1.00	0.800	0.800	0.3020	Uneven	1	4.72	0.92	8[b]
						2	5.73	0.98	8[b]
Methylparaben	1.00	1.000	1.000	0.2508	Rough	1	2.07	0.22	0.80
						2	1.72	0.20	1.00

[a] Method 1 is Brenner's method (modified), average of 12 tablets. Method 2 is the British Pharmacopoeial method (modified), average of 8 tablets.
[b] No disintegration.
[c] Too rough to measure.

TABLE II.—SHELLAC COATINGS

Conspergent	Amt. Soln., Ml./ Coat	Amt. Powd., Gm./ Coat	Ratio, Gm./ Ml.	Thickness, Mm.	Description	Disintegration: Method[a]	Acid Pepsin Soln., Hr.	Alkaline Pancreatin Soln., Hr.	Acid Pancreatin Soln., Hr.
Talc	0.60	0.12	0.200	0.0771	Even	1	5.83	0.25	7.32
						2	1.00	0.27	2.75
Kaolin	0.60	0.41	0.683	0.0571	Uneven	1	1.95	0.18	0.38
						2	0.28	0.13	0.25
Bentonite	0.60	0.60	1.000	0.0944	Even	1	0.42	0.18	0.25
						2	0.43	0.10	0.25
Magnesol	0.60	0.31	0.517	0.0579	Even	1	0.32	0.07	0.22
						2	0.27	0.12	0.25
Magnesium trisilicate	0.60	0.32	0.533	0.0786	Even	1	0.32	0.20	0.50
						2	0.30	0.22	0.25
Tertiary magnesium phosphate	0.60	0.50	0.833	0.0472	Even	1	0.60	0.17	0.38
						2	0.30	0.19	0.28
Magnesium oxide	0.60	0.10	0.167	0.0569	Even	1	3.63	3.00	3.42
						2	0.75	0.40	2.65
Magnesium stearate	0.50	0.36	0.720	0.0871	Even	1	6.55	0.33	2.55
						2	1.88	0.22	1.30
Calcium stearate	0.55	0.52	0.945	0.0886	Even	1	8[b]	0.13	0.18
						2	8[b]	0.17	0.13
Aluminum stearate	1.12	0.84	0.750	0.1983	Even	1	8[b]	1.82	8[b]
						2	8[b]	1.97	8[b]
Aluminum palmitate	0.60	0.28	0.467	0.0866	Even	1	5.92	0.20	1.83
						2	0.85	0.22	0.45
Dry-Flo starch	1.10	1.38	1.236	0.1955	Even	1	5.38	1.38	8.00
						2	4.07	1.05	5.02
Ethyl *p*-aminobenzoate	0.60	2.20	3.667	0.1914	Even	1	1.68	2.75	8[b]
						2	0.75	1.42	4.68
Methylparaben	0.60	1.98	3.300	0.1525	Uneven	1	7.50	1.50	8[b]
						2	3.68	0.58	2.45
n-Propylparaben	0.70	2.50	3.571	0.1121	Even	1	8[b]	3.08	8[b]
						2	8[b]	1.90	8[b]

[a] Method 1 is Brenner's method (modified), average of 12 tablets. Method 2 is the British Pharmacopoeial method (modified), average of 8 tablets.
[b] No disintegration.

ENTERIC COATING OF RED SUGAR COATED PILLS

Stearic Acid, Mutton Tallow, Balsam Tolu, Sodium Taurocholate Coating.—Red sugar coated pills (Upjohn's AC193) were obtained so that the disintegration could easily be determined by the appearance of a pink color in the test solution.

Prior to coating the pills, 25 from each lot were measured with a micrometer and the average diameter calculated. Twenty-five of each lot were measured after coating, and the difference divided by two to obtain the average thickness of the coating (Table I).

The pills were coated in lots of 250 with the formula recommended by Thompson and Lee (6) in 1945. The coating solution contained 8 Gm. of stearic acid, 6 Gm. of mutton tallow, 2 Gm. of balsam tolu, and 1 Gm. of sodium taurocholate in 60 ml. of a mixture of equal volumes of absolute alcohol and acetone. The solution was applied with a medicine dropper, the pills stirred, and when they began to roll freely, a sufficient amount of conspergent was added from an infant syringe to prevent sticking, build up the coating, and hasten drying. After five minutes, cool air from an electric drier was blown across the mouth of the bowl.

The coatings were tested for disintegration in the artificial gastric solution and alkaline pancreatin solution recommended by Toplis (22). An acid pancreatin solution ranging in *p*H from 6.0 to 6.5 was also used. The two methods previously described were employed. The results are shown in Table I.

Shellac Coating of Red Sugar Coated Pills.—The solution employed contained 72 Gm. of arsenic-free shellac in 100 ml. of alcohol. The solution was added from a pipette and the pills stirred with a curved glass rod until they began sticking to the sides of the bowl. A sufficient amount of conspergent was then added to prevent sticking to the sides of the bowl, and the pills stirred with each addition of powder. The pills were dried with air at 30°. Sixteen applications were necessary for satisfactory enteric properties.

The thickness of each coating was determined as previously described. The disintegration of each coating was determined by the two methods described (Table II).

Cetyl Alcohol, Sandarac, Tolu Coatings on Red Sugar Coated Pills.—The third type of enteric coating consisted of the alternate application of two solutions. The first solution contained 45.4 Gm. of cetyl alcohol and 121.6 Gm. of sandarac in 258.6 ml. of absolute alcohol. The second solution contained 227 Gm. of balsam tolu in 143.7 ml. of acetone. Solution was effected by heating on a water bath. The solution was added from a pipette and the pills rolled with stirring until they became tacky, a sufficient amount of conspergent added and the pills dried at 30°. The second solution was then added and the process repeated. After the

two solutions were applied and the pills dried, they were spread on trays and dried overnight at 40°. The process was repeated twice on successive days to give a total of six applications of solution and powder to the pills.

The average thickness of each coating was determined with a micrometer and the disintegration determined in the three artificial digestive juices by the two methods described (Table III).

COATING OF CONVENTIONAL-SHAPED TABLETS

Subcoated tablets of sodium salicylate were obtained from Eli Lilly and Company for the application of the three types of enteric coating solutions with the conspergents which were satisfactory on the red sugar coated pills and the powder recommended for use with each solution.

The coating solutions were applied as previously described with the exception of the stearic acid, mutton tallow solution which was sprayed from an atomizer. An infant syringe was used to apply the conspergent for each solution.

The average thickness of each of the coatings was determined and the disintegration tests conducted by the two methods described (Table IV).

After a short period of storage, the disintegration rates of the enteric coated red sugar coated pills were determined. The values were not sufficiently different from those in the preceding tables to draw definite conclusions.

SUMMARY AND CONCLUSIONS

1. Three kinds of enteric coating solutions were employed with fourteen, fifteen, and sixteen conspergents, respectively.

2. Consideration of the physical properties of the conspergents led to the conclusion that specific gravity and melting point had little influence, and that particle size, crystalline structure, chemical composition, mobility, and solubility may have determined to some extent the results.

3. The amount of powder required for a satisfactory coating varied with the enteric solution and the powder employed. The appearance and behavior of each enteric coating varied with the solution and conspergent.

4. The efficiency of the enteric coatings was not entirely a function of thickness.

5. Magnesium stearate, calcium stearate, talc, and aluminum palmitate were more satisfactory than the other conspergents employed.

TABLE III.—CETYL ALCOHOL, SANDARAC, TOLU COATINGS

Conspergent	Amt., Ml./Coat Soln. 1	Amt., Ml./Coat Soln. 2	Amt. Powd., Gm./Coat	Ratio, Gm./Ml. Soln. 1	Ratio, Gm./Ml. Soln. 2	Thickness, Mm.	Description	Method[a]	Disintegration Acid Pepsin Soln., Hr.	Disintegration Alkaline Pancreatin Soln., Hr.	Disintegration Acid Pancreatin Soln., Hr.
Subcoating powder	1.2	1.50	0.51	0.425	0.340	0.0639	Even	1	4.62	0.25	1.00
								2	0.58	0.22	0.37
Talc	1.5	2.00	1.25	0.833	0.625	0.0678	Even	1	8[b]	4.87	8[b]
								2	8[b]	3.67	3.50
Kaolin	1.5	2.00	0.61	0.407	0.305	0.0581	Uneven	1	8[b]	2.85	2.05
								2	1.22	0.67	0.85
Bentonite	1.5	2.00	1.58	1.053	0.790	0.1244	Uneven	1	0.28	0.15	0.18
								2	0.25	0.13	0.15
Magnesol	1.5	1.97	0.97	0.647	0.492	0.1489	Even	1	6.35	8[b]	8[b]
								2	4.93	3.60	5.48
Magnesium trisilicate	1.5	1.93	1.11	0.740	0.575	0.1058	Uneven	1	0.90	2.83	4.38
								2	0.68	1.00	1.47
Tertiary magnesium phosphate	1.5	1.90	0.85	0.567	0.447	0.1055	Uneven	1	7.32	8[b]	8[b]
								2	1.57	1.97	2.10
Magnesium oxide	1.5	1.90	0.39	0.260	0.205	0.0760	Uneven	1	8[b]	3.12	0.83
								2	1.83	0.92	0.98
Magnesium stearate	1.2	1.60	0.33	0.275	0.206	0.0056	Even	1	8[b]	0.30	0.47
								2	0.73	0.23	0.40
Calcium stearate	1.2	1.60	0.55	0.458	0.363	0.0564	Even	1	8[b]	3.28	3.47
								2	2.43	0.72	0.68
Aluminum stearate	1.5	1.60	0.46	0.307	0.288	0.1849	Very rough	1	8[b]	5.33	8[b]
								2	8[b]	3.30	5.20
Aluminum palmitate	1.2	1.50	0.35	0.292	0.233	0.0788	Very rough	1	5.22	0.23	1.97
								2	0.83	0.40	0.38
Dry-Flo starch	1.5	1.40	0.98	0.653	0.700	0.1432	Even	1	3.72	5.58	5.62
								2	2.02	1.68	1.78
Ethyl *p*-aminobenzoate	1.2	1.20	1.32	1.100	1.100	0.1973	Even	1	5.13	8[b]	8[b]
								2	2.23	8[b]	8[b]
Methylparaben	1.3	1.30	1.43	1.100	1.100	0.1958	Even	1	8[b]	8[b]	5.15
								2	4.78	7.83	6.00
n-Propylparaben	1.5	1.20	1.85	1.233	1.542	0.1196	Uneven	1	8[b]	2.67	8[b]
								2	1.53	2.70	8[b]

[a] Method 1 is Brenner's method (modified), average of 12 tablets. Method 2 is the British Pharmacopoeial method (modified), average of 8 tablets.
[b] No disintegration.

TABLE IV.—ENTERIC COATINGS ON CONVENTIONAL-SHAPED TABLETS

Conspergent	Enteric Soln.[c]	Amt. Ml./Coat Soln. 1	Amt. Ml./Coat Soln. 2	Amt. Powd., Gm./Coat	Ratio, Gm./Ml. Soln. 1	Ratio, Gm./Ml. Soln. 2	Thickness, Mm. Convex Surf.	Thickness, Mm. Edges	Description	Method[a]	Disintegration Acid Pepsin Soln., Hr.	Disintegration Alkaline Pancreatin Soln., Hr.	Disintegration Acid Pancreatin Soln., Hr.
Talc	1	2.45	...	1.102	0.449	...	0.1468	0.1186	Even	1	0.25	0.20	0.13
										2	0.18	0.17	0.07
	2	1.93	...	2.350	1.218	...	0.2160	0.1593	Even	1	2.97	0.25	0.35
										2	0.75	0.10	0.20
	3	2.67	2.67	7.08	2.652	2.652	0.1046	0.1252	Even	1	8.00	3.22	4.48
										2	6.15	1.98	3.60
Magnesium stearate	1	2.60	...	1.088	0.418	...	0.4867	0.1976	Rough	1	3.18	0.30	0.27
										2	0.70	0.50	0.25
	2	1.91	...	0.800	0.419	...	0.2433	0.1012	Even	1	8[b]	0.48	0.63
										2	1.97	0.40	0.60
	3	2.50	2.33	1.285	0.514	0.552	0.1194	0.1696	Even	1	8[b]	4.10	4.57
										2	2.32	0.88	0.80
Calcium stearate	1	2.30	...	1.174	0.510	...	0.4007	0.2641	Even	1	8[b]	0.83	1.52
										2	1.42	0.48	0.78
	2	1.91	...	1.370	0.717	...	0.2986	0.2421	Even	1	4.12	0.30	0.25
										2	2.98	0.15	0.08
	3	2.33	2.33	1.980	0.849	0.849	0.1852	0.2444	Even	1	3.47	3.45	1.03
										2	1.43	0.53	0.70
Aluminum palmitate	1	2.55	...	0.708	0.278	...	0.3177	0.2186	Rough	1	2.33	0.80	2.25
										2	1.55	0.90	1.10
	2	1.91	...	0.840	0.439	...	0.2154	0.1272	Uneven	1	8[b]	0.63	0.80
										2	5.57	0.50	3.30
Subcoating powder	3	2.67	2.67	4.710	1.764	1.764	0.1086	0.0897	Uneven	1	0.97	0.30	0.15
										2	0.22	0.17	0.12

[a] Method 1 is Brenner's method (modified), average of 12 tablets. Method 2 is the British Pharmacopoeial method (modified), average of 8 tablets.
[b] No disintegration.
[c] Solution 1 is stearic acid, mutton tallow, balsam tolu, sodium taurocholate coating. Solution 2 is shellac coating. Solution 3 is cetyl alcohol, sandarac, tolu coating.

6. The number of applications necessary for an enteric coating varied with the substances used. Twenty applications were needed for the stearic acid, mutton tallow coating, sixteen for shellac, and six (three applications of each of the two solutions) for the cetyl alcohol, sandarac, tolu coating.

7. Variation may be noted in the disintegration rates by the two methods. The time required for the coating to break was usually less in the method recommended by the *British Pharmacopoeia*.

8. Shellac coatings were satisfactory on conventional-shaped tablets with talc, magnesium stearate, calcium stearate, and aluminum palmitate; stearic acid, mutton tallow, balsam tolu, sodium taurocholate was satisfactory with calcium stearate; and calcium stearate was the only satisfactory conspergent with the cetyl alcohol, sandarac, tolu coating.

9. The *in vitro* disintegration determinations after storage were not sufficiently different from those obtained on the coatings earlier to be conclusive.

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The Electrophoretic Analysis and Antibacterial Determination of Madronin in Agar Gel*

By E. ROY HAMMARLUND† and L. WAIT RISING‡

By use of an electrophoretic analytical procedure, it was possible to determine the number of ultraviolet absorbing components in a partially purified antibacterial mixture, the extent of their ionic characteristics, and the ultraviolet absorption spectrum of each component. After the electrophoretic separation, the location of the antibacterial substance in the agar diffusion medium could be found by use of a microbiological plating technique.

A MODIFIED method of electrophoretic analysis in agar gel was recently reported by Peniston, *et al.* (1), of the University of Washington. Although the analytical procedure was still in the experimental stage at that time, it was used for the electrophoretic assay of a newly discovered antibacterial substance, madronin, obtained from the fresh leaves of *Arbutus menziesii* (Madrona), family *Ericaceae* (2).

EXPERIMENTAL

The electrophoretic analysis was carried out in a Vycor migration tube, $^3/_4$ x 76 cm., scanned at the absorption peak of 270 mμ, using the method of Peniston, *et al.* (1). The diffusion medium was 0.70% Difco purified agar, and 0.06 *M* acetic acid-sodium acetate buffer of *p*H 5 was used as the solvent for all solution. A sample volume of 1 ml., occupying approximately a 2-cm. length section in the tube was employed for analysis. A voltage gradient of 13.2 v. per cm. was applied, giving a total of 1,000 v.; the average current was 0.033 amp. The temperature of the bath was kept at 18°. A period of five hours was required for the migration time of the sample.

The experimental mobilities obtained were corrected for temperature and for the endosmotic mobility of 8.10×10^{-5} cm.^{2} per volt second at 15° to yield the corrected mobility value in cm.^{2} per volt second at 15°.

The sample to be analyzed was prepared as follows: a 25-mg. sample of madronin was dissolved in 17 ml. of water, and 1 ml. of the solution was added to 9 ml. of acetate buffer. One milliliter of this buffered solution of madronin, containing 0.15 mg. or approximately 3 optical density-cm. units per ml., was placed in the migration tube at a position of 27.5 to 29.6 cm. from the cathode.

Figure 1, which shows the electrophoretic separation of the madronin ingredients, was obtained by plotting the position of the tube in centimeters from the cathode *vs.* the optical density obtained when the entire tube was scanned at 270 mμ, using a Beckman Model DU spectrophotometer.

TABLE I.—ELECTROPHORETIC MOBILITIES OF MADRONIN COMPONENTS

Component	Peak Position[a]	Cm. Migrated	Exptl. Mobility (15°), Cm.^{2}/Volt Sec., $\times 10^{-5}$	Corr. Mobility (15°), cm.^{2}/Volt Sec., $\times 10^{-5}$
1	11.1	−16.9	−6.47	1.63
2	14.2	−14.4	−5.52	2.58
3	19.6	−9.0	−3.45	4.45
4	24.6	−4.0	−1.53	6.57
5	27.8	−0.8	−0.31	7.79
6	29.2	+0.6	+0.23	8.33

[a] Centimeters from cathode.

Table I lists the corrected mobility values for each of the components which appear in Fig. 1. These values were calculated from the experimental mobilities and the temperature correction factor (approximately −3% for each degree of the bath temperature above 15°) as follows:

$$\text{Exptl. mobility} = \frac{\text{(cm. migrated)}\ \text{(temp. correction)}}{\text{(volt gradient/cm.)}\ \text{(migration time, sec.)}}$$

$$\text{Corrected mobility} = \text{experimental mobility} + \text{endosmotic mobility}$$

Figure 2 was obtained by measuring the ultraviolet absorption of the mid point of each of the six peaks in Fig. 1 at wave lengths from 260 and 290 mμ, using a Beckman Model DU spectrophotometer. The values obtained for each component were plotted as optical density units above normal *vs.* wave length.

The reference setting which is used as normal for the spectrophotometer in the above determinations can be any arbitrary position, near either end of the tube, that does not contain any of the ultraviolet absorbing components in question.

Antibacterial Determination of Electrophoresis-Separated Sample.—A method was devised to determine which of the six peaks is composed of the antibacterial substances by microbiologically plating the carefully extruded agar diffusion medium containing the electrophoretically separated components of madronin.

The thin, solid strip of agar from the electrophoresis determination was slowly extruded from the Vycor tube and placed on nutrient agar plates seeded with *Micrococcus pyogenes* var. *albus*. The agar slid easily from the tube onto the plates when

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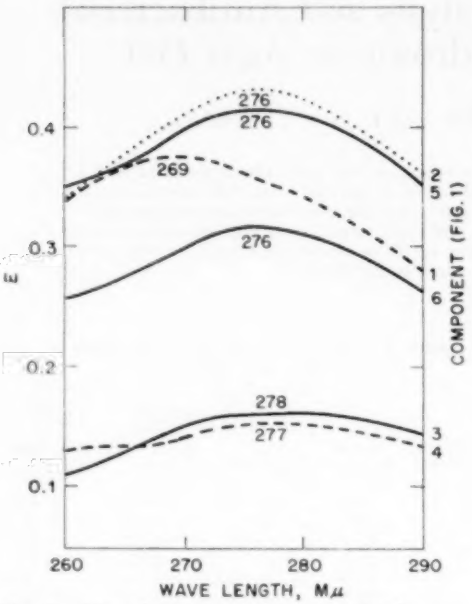

Fig. 2.—Ultraviolet absorption of madronin components.

the open end of the tube was lowered slightly from horizontal, and the flow was controlled and stopped by placing a finger over the opposite end of the tube. The agar strip was pinched off when it had been laid parallel to the first and approximately $1^1/_2$ in. apart. In this manner, it was possible to plate approximately 76 cm. of agar strips in two 6-in. Petri dishes.

To prevent seepage of the liquid from the strip onto the surface of the plate and to insure adequate diffusion of the active material, 50 ml. of *Micrococcus albus* seeded, melted, nutrient agar was then poured on the plates until the parallel strips were embedded and almost covered with the new agar.

The plates were then placed in a refrigerator at 5° for one to two hours to permit diffusion of the antibacterial substances from the electrophoretic agar strip into the seeded nutrient agar. After refrigeration, the plates were incubated at 37° for twenty

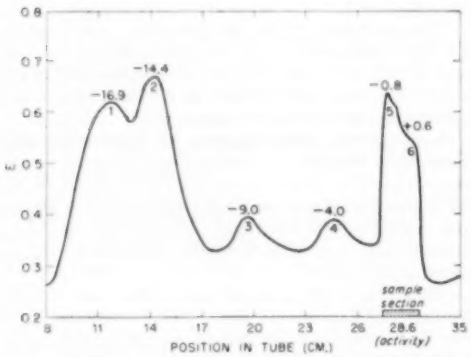

Fig. 1.—Electrophoretic analysis of madronin. Cathode, 0.0 cm.; anode, 76.0 cm.

hours. The appearance of a clear zone in the agar denoted inhibition of bacterial growth, and its distance from one end of the strip was measured with calipers. The exact position of the antibacterial substance was traced on the electrophoretic analysis graph, Fig. 1, and its respective ultraviolet absorption was obtained from Fig. 2.

With this procedure, it was also possible to determine a limited antibacterial spectrum for each of the separated components by making multiple electrophoretic separations and then seeding each series of plates with a different test microorganism. The relative size of the zones of inhibition of growth obtained with each type of microorganism was an indication, within certain limits, of the susceptibility of the microorganisms to each of the separate components of the antibacterial mixture.

The antibacterial spectrum analysis was determined against only a few test microorganisms, since a more complete analysis had already been reported (2). The results of the earlier findings were confirmed in each case. Furthermore, since it had been previously demonstrated (2) that 10% nonhemolyzed beef or horse serum did not decrease or enhance the antibacterial action of madronin tested on agar plates, this test was omitted from the determinations.

DISCUSSION

The electrophoresis graph, Fig. 1, may be interpreted as showing the presence of either four or six separate ultraviolet absorbing components in the madronin mixture. In either case, the substances which compose peaks 1, 2, 3, and 4 are clearly different individual components, as shown by their separate migrations. The difficulty arises in interpreting the presence of peaks 5 and 6.

One possibility is that peaks 5 and 6 are composed of entirely different material from the other four, thus giving a total of six different components. The fact that the only measurable antibacterial activity occurred in this section points to this interpretation. Since the material composing peaks 5 and 6 was still found in the exact position of the sample section after five hours' migration time, it must have resisted migration in some manner. However, the probability is slight that its anionic characteristics were exactly equal to the endosmotic mobility and thereby enabled the material to remain stationary. It is more likely that some insolubility resulted from a reaction between the antibacterial material in peaks 5 and 6 and the buffered agar under the conditions of the test, which would prevent the material from migrating. However, this insolubility was not evident in earlier routine microbiological platings of madronin, when the antibacterial substances easily diffused through the buffered agar medium (2).

Another possibility is that peaks 5 and 6 are composed of the identical substances which comprised peaks 2, 3, or 4, or some combination of them, but for some reason were prevented from complete migration. The fact that peaks 2–6 have nearly identical ultraviolet absorption maxima supports this possibility. Furthermore, if the material which did migrate was too small in quantity to have shown antibacterial activity in the subsequent plate tests, the only activity evident must have come from the region of the stationary peaks 5 and 6.

The second explanation does not satisfactorily fit the pattern of the electrophoresis graph, Fig. 1, since the narrow peaks of the graph do not indicate a slow and slight migration of a single substance. In addition, it is improbable that one part of a substance became insoluble and remained stationary while more of the identical substance migrated freely. Therefore, the interpretation that six separate components were present in the madronin mixture seems to be more probable.

SUMMARY

1. A method is presented for determining the number of ultraviolet absorbing components present in a partially purified antibacterial mixture by use of electrophoretic analysis in agar gel.

2. The ionic characteristics and the ultraviolet absorption spectrum of each of the components are given.

3. The location of each antibacterial substance in the diffusion medium after electrophoresis is determined by a microbiological plating technique.

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A Pharmacological Study of a Series of Diethylaminoethyl Esters of Disubstituted Malonates*

By CECIL P. HEADLEE† and LEROY D. EDWARDS‡

Certain basic esters of substituted malonic acid have been studied for spasmolytic activity against ileal spasms produced by Mecholyl and barium. The results of the screening investigation indicate that all the compounds are to some degree spasmolytic and that the 2-diethylaminoethyl ethyl ester hydrochloride of isoamylethylmalonic acid is one of the more active members of the series. Further studies show that this compound possesses local anesthetic properties. There was no evidence of antihistaminic or prolonged depressor activities.

MANY ATTEMPTS have been made to produce a compound which possesses both neurotropic and musculotropic spasmolytic properties to somewhat the same degree. Nine new compounds, designed to possess such activity, have recently been synthesized by McBay, Jenkins, and Data (1) and have been submitted to this laboratory for evaluation. Chemically these compounds are basic esters of substituted malonic acid. Four 2-diethylaminoethyl ethyl ester hydrochlorides of dibenzylmalonic, benzyl-*n*-butylmalonic, benzylethylmalonic, and isoamylethylmalonic acids, and five bis-2-diethylaminoethyl ester dihydrochlorides of dibenzylmalonic, benzyl-*n*-butylmalonic, isoamylethylmalonic, di-*n*-hexylmalonic, and diallylmalonic acids have been prepared. For brevity, serial numbers D-201 to D-209 have been employed to designate the compounds. The names of the compounds with their corresponding serial numbers are listed as follows:

Ethyl-2-diethylaminoethyl dibenzylmalonate hydrochloride	D-201
Ethyl-2-diethylaminoethyl benzyl-*n*-butylmalonate hydrochloride	D-202
Ethyl-2-diethylaminoethyl ethylbenzylmalonate hydrochloride	D-203
Ethyl-2-diethylaminoethyl ethylisoamylmalonate hydrochloride	D-204
Bis-2-diethylaminoethyl dibenzylmalonate dihydrochloride	D-205
Bis-2-diethylaminoethyl ethylisoamylmalonate dihydrochloride	D-206
Bis-2-diethylaminoethyl di-*n*-hexylmalonate dihydrochloride	D-207
Bis-2-diethylaminoethyl diallylmalonate dihydrochloride	D-208
Bis-2-diethylaminoethyl benzyl-*n*-butylmalonate dihydrochloride	D-209

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EXPERIMENTAL

Screening Test for Antispasmodic Action

Isolated Intestine.—The spasmolytic action of the group of new compounds was determined on isolated guinea-pig ileum using the method of Magnus (2). Mecholyl bromide and barium chloride were employed as the spasmogens and doses used routinely throughout the study were 1 μg. and 5 mg./75-cc. bath, respectively. All the compounds were tested in dilutions up to 0.4 mg./75-cc. bath. Papaverine, atropine, and Trasentine were used as standards. The results in Table I indicate that although the compounds are more effective than papaverine or Trasentine against the musculotropic type of spasm, none is as effective against neurotropic spasm, as either atropine or Trasentine. In view of the fact that D-204 showed the most spasmolytic action against the Mecholyl-induced spasms and considerable spasmolytic action against the barium-induced spasm, this compound was considered the most promising of the group and worthy of further study.

TABLE I.—RELATIVE ANTISPASMODIC POTENCIES OF SEVERAL BASIC ESTERS OF SUBSTITUTED MALONIC ACID USING GUINEA-PIG ILEUM IN A 75-ML. BATH AS THE TEST OBJECT[a]

Drug	Spasmolytic Action—Musculotropic Effect, %	Spasmolytic Action—Neurotropic Effect, %
Papaverine	100.0	...
Atropine	...	100.00
Trasentine	14.9	2.30
D-201	361.0	0.05
D-202	532.0	0.09
D-203	237.0	0.04
D-204	368.0	0.20
D-205	519.0	0.08
D-206	227.0	0.02
D-207	>167.0	>0.03
D-208	286.0	>0.03
D-209	368.0	0.07

[a] The value of unity is arbitrarily given to the standards.

Pharmacology of D-204

Acute Toxicity.—The acute toxicity of D-204 was determined in albino mice by intravenous injection. The drug was dissolved in normal saline and a total volume per mouse of 0.25 cc. was injected. Fatal doses produced a very rapid course of action. All deaths were preceded by violent clonic convulsions, general paralysis, and collapse. Deaths appeared to be due to respiratory failure. The LD_{50} determined on 100 animals and calculated by De Beer's method (3) was 26.5 ± 0.1027 mg./Kg. Death occurred in thirty-six hours.

Antispasmodic Effect.—Compound D-204 was studied further for its spasmolytic properties, using isolated rabbit ileum suspended in an aerated Locke-Ringer's solution. The method of Magnus (2) was employed. Trasentine was selected as the standard and Mecholyl bromide and barium chloride as the spasmogens. Data presented in Table II demonstrate that D-204 was approximately 18 times more potent than Trasentine against the barium-induced spasms but showed only 0.03 times the potency of the standard when tested against the Mecholyl-induced spasms.

TABLE II.—ANTISPASMODIC ACTIVITY OF D-204 AS TESTED IN VITRO ON ISOLATED RABBIT INTESTINE

Drug	Amt. Drug to Decrease Spasms 50%, Tested Against: $BaCl_2$, 5 Mg./75-Cc. Bath	Amt. Drug to Decrease Spasms 50%, Tested Against: Mecholyl Br., 1 μg./75-Cc. Bath	Deg. Activity Tested Against[a]: $BaCl_2$, 5 Mg./75-Cc. Bath	Deg. Activity Tested Against[a]: Mecholyl Br., 1 μg./75 Cc. Bath
D-204	0.25 mg.	130.0 μg.	18.00	0.03
Trasentine	4.50 mg.	4.2 μg.	1.00	1.00

[a] Unity is arbitrarily assigned to Trasentine.

Inhibition of Senna Diarrhea.—Oral administration of D-204 markedly decreased the incidence of diarrhea in senna-treated mice. The method used in the experiment was that as modified by Hazleton and Fortunato (4). A suspension of standard Tinnevelly senna powder at a dose level of 500 mg./Kg., administered orally to 100 albino mice, produced diarrhea. Results of the experiment (see Table III) indicate that in doses of 10 mg./Kg., the inhibitory effect of D-204 is about the same as that shown by Trasentine, the standard. At a dosage of 50 mg./Kg., however, the new ester shows an activity 14% greater than that of Trasentine.

TABLE III.—EFFECTS OF D-204 AND TRASENTINE IN MICE TREATED WITH 500 MG./KG. OF SENNA POWDER

Dose, Mg./Kg.	Incidence of Diarrhea, %—Control	Incidence of Diarrhea, %—D-204	Incidence of Diarrhea, %—Trasentine
10	80	70	70
50	80	40	55

Antihistamine Activity.—The action of histamine on the blood pressure of anesthetized cats (5) was not blocked by doses of D-204 up to 9.6 mg./Kg., nor did the new ester, over a dose range of 5–80 mg./Kg., protect guinea pigs (6) against a histamine aerosol.

Local Anesthetic Action.—Topical anesthesia as produced by D-204 was investigated by instilling aqueous solutions of the test drug into the conjunctival sacs of albino rabbits. Infiltration anesthesia was studied by injecting doses of the test drug, dissolved in normal saline, intracutaneously into the backs of guinea pigs. The duration (thirty minutes) of the topical anesthesia produced by a 0.25% solution of D-204 was approximately equal to that of a 1.0% solution of cocaine hydrochloride. When injected intracutaneously, the local anesthesia of a 1.0% solution of cocaine was duplicated by a 1.0% solution of D-204.

Irritation.—The instillation of a 3% solution of D-204 into the rabbit's conjunctival sac produced severe irritation consisting of corneal opacity, beefy redness of the conjunctiva, a severe swelling of the eyelids and considerable discharge. This irritation lasted up to seventy-two hours but no residual injuries occurred. With a 2% solution, there was less edema and inflammation and far less discharge. Instillation of a 1% solution produced a slight temporary inflammation and discharge but little or no edema. Concentrations of 0.5% or less produced no observable symptoms of irritation. With concentrations of 1% and greater, there was a slight dilation of the pupil; the light reflex was also somewhat inhibited.

The intracutaneous injection of 0.25 cc. of a 0.5% solution, using guinea pigs, produced little or no irritation as evidenced by tissue damage. Concentrations above 0.5% produced tissue damage of increasing severity as the strength of the solution was increased. The most severe irritations, produced by a 5% solution, healed in two to three weeks with scar formation.

Action on the Cardiovascular and Respiratory Systems.—Intravenous injections of D-204 into the barbitalized cat in a dose of 0.5 mg./Kg. caused a transient fall in arterial blood pressure and a slight increase in the depth of respiration. This drop in blood pressure was very rapid but a return to normal was effected in one and one-half minutes or less. The hypotensive effect of D-204 increased in degree and duration as the dose of the drug was increased. With the six cats used, death was produced at approximately 20 mg./Kg. Upon injection of the fatal dose there was a cessation of the heart beat almost simultaneously with that of respiration. The over-all effect on respiration was one of stimulation with low doses and depression with higher doses. Doses of 4.0 mg./Kg. produced an increase in both rate and depth; at the 8-mg./Kg. level, there was a decrease in rate but an increase in depth, while at doses of 12 mg./Kg. and above, both rate and depth were decreased.

The blood pressure of rats made hypertensive by a modified Goldblatt technique (7) was only momentarily lowered by intravenous doses of D-204 up to 30 mg./Kg.

CONCLUSIONS

1. A series of nine basic esters of substituted malonic acid has been screened for spasmolytic activity, using the spastic guinea-pig ileum method. All the compounds were highly active against the musculotropic type of spasm but were weakly active against the neurotropic type of spasm.

2. Ethyl-2-diethylaminoethyl ethylisoamylmalonate hydrochloride, most active of the group against both types of spasms, was approximately 18 times more active than Trasentine against the musculotropic spasm but was only 0.03 times as active as Trasentine against a neurotropic spasm.

3. The LD_{50} of D-204, determined intravenously in albino mice, was 26.5 ± 0.1027 mg./Kg.

4. The antidiarrhetic effect of D-204 was found to be 14 per cent greater than that of Trasentine.

5. Tests indicate that D-204 is effective as a local anesthetic.

6. Concentrations of D-204 of 0.5 per cent or less show little irritation topically or intracutaneously.

7. Tests for antihistaminic and prolonged depressor activities were essentially negative.

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The Reduction of Schiff Bases with Lithium Aluminum Hydride*

By RAYMOND N. CASTLE, DUANE L. ALDOUS, and MINETTE HALL

The reduction of Schiff bases with lithium aluminum hydride has been studied. Two compounds were reduced which contained an ester functional group in addition to the azomethine linkage. These were reduced smoothly to the benzylaminobenzyl alcohols.

THE PURPOSE of this study was to prepare substituted benzylamines which might possess physiological activity. The lithium aluminum hydride reduction of Schiff bases appeared to offer a good synthetic route to these compounds. The chemistry and scope of this versatile reducing agent has recently been reviewed (1). In a paper by Nystrom and Brown (2) the reduction of benzylidene aniline to N-benzylaniline was reported. This reduction has been confirmed in the present investigation.

N-(2-hydroxybenzyl)-*p*-toluidine has been prepared by the reduction of 2-hydroxybenzylidene *p*-toluidine with sodium amalgam (3). Emmerich reported a melting point of 116° for this compound by his method. N-(2-hydroxybenzyl)-

* Received December 23, 1952, from the Laboratory of Pharmaceutical Chemistry, The University of New Mexico, Albuquerque, N. Mex.

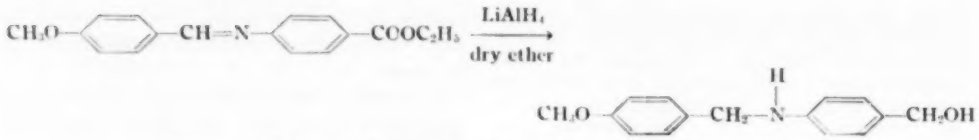

Fig. 1.—Reduction of 4-methoxybenzylidene-4′-carbethoxy aniline.

p-toluidine has been prepared by the lithium aluminum hydride reduction of the Schiff base in 33 per cent yield. The compound thus obtained melts at 122–123°. Likewise Emmerich (4) has prepared N-(2-hydroxybenzyl)-β-naphthylamine by the sodium amalgam reduction of 2-hydroxybenzylidene-β-naphthylamine. N-(2-hydroxybenzyl)-β-naphthylamine has been prepared by the lithium aluminum hydride reduction of the Schiff base in 36 per cent yield. Our compound thus obtained melts at 155° in contrast to the value of 147° reported by Emmerich. N-(2-hydroxybenzyl)-*m*-toluidine was prepared by the lithium aluminum hydride reduction of 2-hydroxybenzylidene-*m*-toluidine in 55 per cent yield.

In order to reduce the azomethine linkage simultaneously with another functional group known to be reducible with lithium aluminum hydride, the reduction of two Schiff bases prepared from ethyl *p*-aminobenzoate (benzocaine) was selected. 2-Hydroxybenzylidene-4′-carbethoxy aniline was reduced to 2-hydroxybenzylaminobenzyl alcohol in 40 per cent yield. Likewise 4-methoxybenzylidene-4′-carbethoxy aniline was reduced to the 4-methoxybenzylaminobenzyl alcohol in 69 per cent yield. Thus the lithium aluminum hydride reduction of carbethoxylated Schiff bases appears to offer a convenient method for the preparation of these hitherto unknown benzylaminobenzyl alcohols. Of the five compounds prepared by the lithium aluminum hydride reduction of the Schiff bases, only those prepared by Emmerich have previously been recorded in the literature.

Figure 1 indicates the course of the reduction of 4-methoxybenzylidene-4′-carbethoxy aniline to 4-methoxybenzylaminobenzyl alcohol. The other reductions are analogous.

EXPERIMENTAL

N-(2-hydroxybenzyl)-*m*-toluidine.—To a stirred slurry of 1.52 Gm. (0.04 mole) of lithium aluminum hydride in 100 ml. of absolute ether was added 8.46 Gm. (0.04 mole) of 2-hydroxybenzylidene-*m*-toluidine in 150 ml. of absolute ether during a fifteen minute period. The mixture was refluxed for an additional forty minute period and then hydrolyzed by the cautious addition of water. The ether layer was separated, dried over anhydrous magnesium sulfate, a part of the ether evaporated, and the compound allowed to crystallize. The yield was 4.65 Gm. (55%). The sample purified for analysis by recrystallization from ethanol melted at 122°.

Anal.—Calcd. for $C_{15}H_{14}NO$: N, 6.57. Found: N, 6.34.

N-(2-hydroxybenzyl)-*p*-toluidine. — This compound was prepared from the corresponding Schiff base as described above. The yield was 33%. The compound when purified for analysis by recrystallization from ethanol melted at 122–123°.

Anal.—Calcd. for $C_{15}H_{14}NO$: N, 6.57. Found: N, 6.45.

N-(2-hydroxybenzyl)-β-naphthylamine. — This compound was prepared from the corresponding Schiff base as described above. The yield was 36%. The compound when purified for analysis by recrystallization from ethanol melted at 155°.

Anal.—Calcd. for $C_{17}H_{15}NO$: N, 5.62. Found: N, 5.55.

4-Methoxybenzylaminobenzyl Alcohol. — This compound was prepared from the corresponding Schiff base as described above except that the following proportions of reactants were used: 0.06 mole of lithium aluminum hydride, and 0.02 mole of the Schiff base. These molar proportions were used in order to reduce the carbethoxy group simultaneously with the azomethine linkage. The yield was 69%. A sample purified for analysis by recrystallization from ethanol melted at 56°.

Anal.—Calcd. for $C_{15}H_{17}NO_2$: N, 5.76. Found: N, 5.73.

2-Hydroxybenzylaminobenzyl Alcohol. — This compound was prepared from the corresponding Schiff base as described above. The yield was 40%. A sample purified for analysis by recrystallization from ethanol melted at 110°.

Anal.—Calcd. for $C_{14}H_{15}NO_2$: N, 6.11. Found: N, 5.83.

SUMMARY

Five Schiff bases have been reduced to the corresponding substituted benzylamines with lithium aluminum hydride. Two of these compounds containing a carbethoxyl group were simultaneously reduced to the corresponding benzylaminobenzyl alcohols. The method gives compounds of a high degree of purity.

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Medicinal Derivatives of *p*-Tertiarybutylbenzoic Acid*,†

By J. T. BRYAN,‡ W. M. LAUTER, and P. A. FOOTE

Fifteen esters of *p*-tertiarybutylbenzoic acid with amino alcohols were synthesized, and their physical and chemical constants were determined. The rearrangement of the corresponding amide to β-ethylaminoethyl-*p*-tertiarybutylbenzoate was shown to proceed with greater ease than the procedure previously described for similar amides obtained from secondary amino alcohols.

THERE have been several papers published from this College reporting derivatives of a substituted benzoic acid, namely, *p*-isopropyl benzoic acid synthesized in the hope of producing useful medicinal or pharmaceutical products. Interest in a newly available derivative of benzoic acid can thus be readily understood.

p-Tertiarybutylbenzoic acid,[1] $C_{11}H_{14}O_2$, (mol. wt. 178.11) was apparently produced for the first time by Pahl (1) in 1884. It is a white, crystalline solid and has a reported low toxicity. Although this acid has been known for many years, no reports have been published on any of its derivatives which may have medicinal or pharmaceutical value.

The *p*-tertiarybutylbenzoic acid used in this investigation needed no additional purification before use. It was a colorless, crystalline solid with a melting point of 160–161°.

As one phase of this study, a series of esters of amino alcohols containing from one to five methylene groups in the alkyl portion of the molecule were synthesized. Pharmacological studies have been conducted on these compounds for use as local anesthetics and antispasmodics (9).

The Schotten-Baumann reaction was chosen as the most adaptable reaction for esterification of the amino alcohols. The preparation of the acid chloride was carried out by using a large excess of thionyl chloride. The thionyl chloride was purified by the procedure recommended by Fieser (2). Purification of the acid chloride by distillation had to be discontinued because of decomposition on heating to temperatures sufficient for distillation. Thus, it was felt that removal of the excess thionyl chloride by distillation was sufficient purification at this point of the synthesis.

The amino alcohols used in this study were obtained either commercially or by synthesis. The following were synthesized in this laboratory: diethylaminomethanol, diethylamino-*n*-butanol, piperidinoethanol, morpholinoethanol, and 2-dibenzylaminoethanol.

EXPERIMENTAL

p-Tertiary-Butyl-Benzoyl Chloride.—One hundred grams (0.56 mole) of *p*-tertiarybutylbenzoic acid was refluxed for six hours with 150 Gm. (1.2 mole) of thionyl chloride. After the reaction was complete, a clear amber-colored liquid remained in the flask. The excess thionyl chloride was removed by distillation under reduced pressure, using a water aspirator at approximately 35 mm. The acid chloride boiled at 266–268° with decomposition. In order to establish definitely that *p*-tertiarybutylbenzoyl chloride had been synthesized, *p*-tertiarybutylbenzamide was prepared by adding 5 Gm. (0.025 mole) of acid chloride dropwise to 10 cc. of 28% ammonium hydroxide; the reaction was immediate and vigorous. The white, solid amide formed was filtered off and washed with water until no odor of ammonia remained; it was then crystallized from hot ethanol. The crystals were dried at 100° in an oven for two hours. The melting point was determined as 170–171°, a value corresponding to that reported by Kelbe and Pfeiffer (3).

Esters of p-Tertiarybutylbenzoic Acid.—All of the esters in this study, with the exception of 2-dibenzylaminoethyl - *p* - tertiarybutylbenzoate and methyl-*p*-tertiarybutylbenzoate, were prepared by methods which can be typified by the preparation of β-dimethylaminoethyl-*p*-tertiarybutylbenzoate. Thirty-six grams (0.41 mole) of β-dimethylaminoethyl alcohol was reacted with 79 Gm. (0.39 mole) of *p*-tertiarybutylbenzoyl chloride in the presence of 200 cc. of 10% aqueous sodium hydroxide solution. The reaction was immediate and external cooling was necessary; the solution was agitated by an electric stirrer. After the esterification was complete (approximately thirty minutes) the oily ester separated and was removed from the mixture. All excess alkali was removed by washing with water. The ester was dried over anhydrous sodium sulfate for twelve hours, and was then fractionated under reduced pressure. Physical constants of the esters are recorded in Table I.

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† Abstracted in part from a thesis submitted by J. T. Bryan to the Graduate School of the University of Florida in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
‡ Fellow of the American Foundation for Pharmaceutical Education.
[1] This acid is currently being produced by the Shell Development Co. from petroleum raw products and is available in sufficient quantities for laboratory and commercial use.

TABLE I.—ESTERS OF *p*-TERTIARYBUTYLBENZOIC ACID

Radicals	d_{25}^{25}	n^{25}	B. P., °C. at 1 mm.	M. P., °C. (Corr.)	Molar Refraction—Calcd.	Molar Refraction—Found	Yield, %
Diethylaminomethyl[a]	....	....	.....	55–56	....	....	56.5
β-Diethylaminoethyl	0.9961	1.5040	153–154	...	83.02	87.48	91.4
β-Dimethylaminoethyl	0.9949	1.5050	143–144	...	73.73	74.32	64.7
γ-Diethylaminopropyl	0.9666	1.4970	157–158	...	87.66	88.14	74.5
β-Diethylaminopropyl	0.9776	1.5116	144–145	...	87.67	89.23	38
Diethylamino-*n*-butyl	0.9891	1.5082	142–143[d]	...	92.23	91.87	89
1-Diethylamino-4-pentyl	0.9965	1.5093	170–171	...	96.96	95.61	41.5
Piperidinoethyl	1.0180	1.5149	158–160	...	85.67	85.67	79.4
Morpholinoethyl	1.0667	1.5210	167–168	...	82.78	83.08	61
2-Dibenzylaminoethyl	1.0409	1.5508	255 (dec.)	...	123.4	122.8	73
β-Ethylaminoethyl	1.0417	1.5240	158–160	...	73.59	73.16	76
N-methyl-diethylamino-bis	1.0681	1.5339	273 (dec.)	...	127.65	127.90	50
N-*n*-Butyl-diethylamino-bis	1.0440	1.5221	190 (dec.)	...	141.70	142.81	61.2
N-phenyl-diethylamino-bis[b]	....	....	.....	104–105	....	....	12.5
Methyl[c]	1.0113	1.5102	105–106[e]	...	56.03	56.83	95

[a] Nitrogen determination, % (Kjeldahl): Calcd., 5.31. Found, 5.61.
[b] Nitrogen determination, % (Kjeldahl): Calcd., 2.78. Found, 2.82.
[c] Ester value: Calcd., 192.1. Found, 192.4.
[d] At 3 mm. pressure.
[e] At 2 mm. pressure.

Methyl-*p*-tertiarybutylbenzoate was prepared by the conventional method of esterification between an alcohol and acid, using sulfuric acid as a catalyst. 2-Dibenzylaminoethyl-*p*-tertiarybutylbenzoate was prepared by dissolving 25 Gm. (0.11 mole) of 2-dibenzylaminoethanol in 175 cc. of pyridine, adding 20 Gm. (0.11 mole) of *p*-tertiarybutylbenzoyl chloride, and stirring with an electric stirrer until solution was effected. The mixture was then refluxed for five hours and was poured into 1 L. of water. This aqueous mixture was extracted with 200 cc. of diethyl ether. After separation the ethereal solution was treated with 100 cc. of 10% hydrochloric acid, separated, and shaken with 50 cc. of 5% sodium carbonate solution. The ether was evaporated, and the ester was fractionated under reduced pressure. Physical constants are shown in Table I.

Amino Alcohols.—Diethylaminomethyl alcohol was prepared by the method of Henry (4). Diethylamino-*n*-butyl alcohol was synthesized by the procedure of Starr and Hixon (5) in which tetramethylene chlorohydrin was first prepared from tetrahydrofuran. The chlorohydrin was then condensed with diethylamine. Piperidinoethyl alcohol and morpholinoethyl alcohol were prepared by similar methods. Equimolar quantities of the amine and ethylene chlorohydrin were refluxed for two hours on a steam bath. The mixture was contained in a two-necked flask and the ethylene chlorohydrin was added to 300 cc. of 30% aqueous sodium hydroxide solution and stirred vigorously with an electric stirrer. The amino alcohol, which separated as an oily layer, was extracted from the aqueous mixture with 200 cc. of diethyl ether. The ether was evaporated and the alcohol was fractionated under reduced pressure. The 2-dibenzylaminoethyl alcohol was synthesized by the method of Gump and Nikawitz (6).

Salts of the Esters.—The hydrochlorides were prepared by passing dry hydrogen chloride into the respective esters, using diethyl ether as a solvent. The resulting solid hydrochlorides were purified by crystallization from ethanol. The per cent of chlorine in each compound was determined gravimetrically by the silver method and was compared to the theoretical value (Table II). The oxalates and the maleate were formed by adding ethereal solutions of the esters to saturated solutions of the respective acids in diethyl ether. The solids were crystallized from hot ethanol.

TABLE II.—SALTS OF ESTERS OF *p*-TERTIARYBUTYLBENZOIC ACID

Radicals	M. P.[c] °C.	Nitrogen,[a] %—Calcd.	Nitrogen,[a] %—Found	Chlorine,[b] %—Calcd.	Chlorine,[b] %—Found	Yield, %
β-Diethylaminoethyl HCl	154–155	4.47	4.42	11.31	11.35	66
β-Dimethylaminoethyl HCl	183–184	4.91	5.01	12.41	12.47	73.4
γ-Diethylaminopropyl HCl	188–189	4.27	3.91	10.82	10.77	59.4
β-Diethylaminopropyl HCl	179–180	4.27	4.24	10.82	10.87	72.4
Diethylamino-*n*-butyl HCl	79–98	4.10	3.90	10.39	10.34	80
1-Diethylamino-4-pentyl Oxalate	127–129	3.14	3.16	...	...	78
Piperidinoethyl HCl	193–194	4.29	4.17	10.88	11.20	71
Morpholinoethyl HCl	195–196	4.27	4.15	10.82	11.13	70
2-Dibenzylaminoethyl HCl	181–182	3.19	3.09	8.07	7.91	62.5
β-Ethylaminoethyl HCl	178–179	4.89	4.81	12.42	12.03	41.5
N-methyl-diethylamino-bis maleate	168–169	2.52	2.53	...	...	79.4
N-*n*-butyl-diethylamino-bis oxalate	121–122	2.45	2.51	...	...	25.2

[a] By the semimicro Kjeldahl method.
[b] By the gravimetric method.
[c] Corrected melting points.

Rearrangement of N-β-Hydroxyethyl-p-Tertiarybutylbenzamide to β-Ethylaminoethyl-p-tertiarybutylbenzoate.—It has been shown by Hancock and Cope (7) that an amide is formed when secondary amino alcohols are used in the Schotten-Baumann reaction. This fact was confirmed by Reasenberg and Goldberg (8) and was found to hold true in this instance:

$$(C_4H_9)C_6H_4COCl + HOCH_2CH_2NH(C_2H_5) \longrightarrow (C_4H_9)—C_6H_4CON(C_2H_5)—CH_2CH_2OH$$

$$\xrightarrow{\text{heat}} (C_4H_9)—C_6H_4COOCH_2CH_2NH—(C_2H_5)$$

After the reaction in which the amide was formed, the tertiary nitrogen was shown to be neutral when it failed to react with strong mineral and organic acids to form salts.

In order to effect rearrangement of the amide to the amine by the method of Hancock and Cope (7), the amide was treated with concentrated hydrochloric acid and heated for five minutes; it was then poured into water and the excess acid was neutralized with ammonium hydroxide. After this treatment, an oil separated from the mixture and was isolated. The oil readily formed both an oxalate and a hydrochloride.

In an attempt to purify the amide by vacuum distillation, it was found that complete conversion to the amine took place, confirmed by the melting point. Therefore heat alone is sufficient to rearrange the molecule, and no strong mineral acid is required.

SUMMARY AND CONCLUSIONS

A number of esters of p-tertiarybutylbenzoic acid have been synthesized. With the exception of methyl-p-tertiarybutylbenzoate, all of the esters contained an amino alcohol as a component part of the molecule. All liquid esters were converted to solids by formation of the respective hydrochloride, oxalate, or maleate.

The following physical and chemical constants were determined for the compounds: boiling point, specific gravity, refractive index, and molar refraction for the liquids; melting point, nitrogen determination, and chloride determination for the solids.

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Solution Time of Hypodermic Tablets*

By E. L. WALTERS, G. L. CHRISTENSON, and H. R. KREIDER, Jr.

An apparatus for determining dissolution rates of soluble tablets is described. The importance of control of agitation in dissolution rate determination is shown. The instrument described can be closely controlled in this factor, and dissolution rate results achieved with it are more accurate and reproducible than those obtainable when operator judgment governs inversion rate.

NUMEROUS papers have appeared in the literature reporting different methods of determining the disintegration time of compressed tablets, sugar coated tablets, and enteric coated tablets. Pieces of apparatus have been described which can be used for this purpose (1–4), as well as for many others.

A large-scale study has been carried out by a Subcommittee of the Combined Contact Committee of the A. D. M. A. and A. PH. M. A. and, the Stoll-Gershberg apparatus has now been selected as the official U. S. P. method. This study included only compressed tablets which disintegrate in the presence of water or artificial gastric juice and yield granules of the original mixture, before compression, sufficiently small to pass through a No. 10 screen. It is ob-

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TABLE I.—DISSOLUTION RATES IN SECONDS OF 1/4-GR. SACCHARIN TABLETS BY THE MACHINE METHOD

	Product A		Product B		Product C	
	Operator A	Operator B	Operator A	Operator B	Operator A	Operator B
Arith. average[a]	68	60.6	30	31.4	9.7	8.0
Maximum deviation	20	9.6	15	9.6	2.3	3.0
Average deviation	9.2	5.24	6.4	3.76	0.96	1.0

[a] 20 tablets.

vious that this method does not necessarily determine the time of complete dissolution because particles smaller than No. 10 mesh will also pass through the screen.

Hypodermic tablets are usually made by diluting the active ingredient with a completely soluble excipient (such as lactose or lactose-sucrose mixtures), moistening the mixture with an alcoholic-water solution to dissolve a small portion of the powders, molding to shape, and drying. The material in solution crystallizes upon evaporation of the solvent and forms bridges between the particles so that the mass is sintered together as a single porous tablet. When hypodermic tablets are placed in water the water dissolves these crystals and the tablet disintegrates by simple dissolution. Complete clarity must be attained before the solution may be used for injection.

TABLE II.—DISSOLUTION RATE IN SECONDS OF 1/2-GR. MORPHINE H. T. NO. 39285 BY THREE DIFFERENT METHODS

	Routine	Standardized Routine[a]	Machine Method
	8	25	35
	9	35	42
	6	26	33
	6	23	42
		18	42
Av.	7.25	25.4	40.2

[a] Complete inversion of tube every 2 sec. 6-in. test tube, 10 cc. of H_2O at 25°.

Thus, a very desirable characteristic of hypodermic tablets is rapid dissolution in a relatively small amount of water. In carrying out work on improving the formulation and the manufacture of this class of tablets, it was apparent to the authors that an apparatus was needed to determine the time of solution under standard conditions of temperature, solution volume, and agitation so that the modifications under consideration could be evaluated accurately.

EXPERIMENTAL

The Stoll-Gershberg apparatus was tried with six tablets, as specified in the U. S. P. XIV. Tablets fell through the screen in 20–40 seconds and then rested on the bottom of the beaker for some time without going into solution. Since changing the screen and otherwise varying the agitation would entail major modification and re-evaluation of the apparatus, it was desirable to find a simpler method which would correlate with actual conditions of use, employ a minimum of agitation, and give a clearly visible end point.

An apparatus was designed which met these conditions (Fig. 1), consisting of a test tube 15 cm. in length and 15 mm. in diameter, bent to a 150° angle 9 cm. from the bottom of the tube. Perpendicular to and at the bend was attached a short piece of glass tubing which was held in a chuck on a synchronous motor rotating at 20 r. p. m. This speed was chosen by trial to give a sharp visual end point.

In actual test, 10 ml. of distilled water at 25° was added and the tube was attached to the chuck with the open end on a horizontal plane. The tablet was placed on the flat side at the open end of the tube and the tube was stoppered. The motor was started and the time of complete solution was measured by a stop watch. The end point is the time required for complete solution, determined by visual inspection.

In the preliminary evaluation of this instrument, molded saccharin tablets from three sources were tested by two operators to determine the variations in operator technique as well as the individual tablet

TABLE III.—VARIATION IN DISSOLUTION RATES IN SECONDS BY OPERATOR AND METHOD, USING 1/2 GR. OF MORPHINE H. T. NO. 39377

	Operator A		Operator B		Operator C		Operator D		Operator E		Operator F	
	Routine Method	Machine Method	Routine Method	Machine Method	Routine Method	Machine Method	Routine Method	Machine Method	Routine Method	Machine Method	Routine Method	Machine Method
Arith. av.[a]	22.2	43.6	39.9	40.4	30.2	41.5	81.6	40.4	59.9	39.9	21.1	38.2
Max. dev.	10.2	11.4	12.1	15.4	5.8	9.1	20.8	8.8	13.7	15.5	2.3	11.2
Av. dev.	5.0	4.9	8.5	6.7	2.7	5.5	8.7	4.3	5.5	7.3	1.3	4.6

[a] 20 tablets.

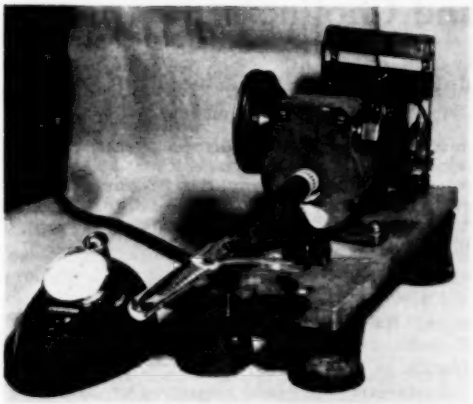

Fig. 1.—Apparatus for rate of tablet dissolution.

variation. Twenty tablets were used in each experiment. These data are shown in Table I and indicate that the instrument gave uniform results from operator to operator on any one lot of tablets. This is especially true with the more rapidly disintegrating tablets.

By further screening of methods available to the authors, the data shown in Table II were obtained. These preliminary individual dissolution rates indicated that considerable discrepancy between the two methods could be expected. Very rapid rates are obtained by the routine method in this series which consists simply of adding water and tablet to a test tube and inverting until dissolution is obtained. Since such factors as volume per tube, temperature, and inversion rate are not controlled, the method is inaccurate and not reproducible from operator to operator, as will be shown later. The data in Table II also indicate that a standardized hand inversion technique gives results comparable to the standardized machine method. By variation in speed of rotation or inversion, the two standardized methods can be made to give the same results. The advantage of the machine method lies in the elimination of the human element in regulating the inversion rate and obtaining reproducible mixing.

To check the preliminary findings further, the study was expanded to include six operators; results are shown in Table III. All of the people chosen were familiar with the routine method previously employed in this laboratory. They were asked to determine the dissolution rate of $^1/_2$-gr. morphine sulfate hypodermic tablets by the routine method and by the standard machine method. Twenty tablets were used in each determination. Table III clearly shows that an operator will not deviate too far from his own average, but differences in averages from one operator to another demonstrate the lack of reproducibility with the "routine" method. The results obtained by all six operators using the standard machine method indicate that the important factors in the measurement of the solution rate of soluble tablets have been controlled, since constant reproduction by various operators has been established. These facts are illustrated more clearly in Table IV, definitely indicating that the machine method eliminates the human error inherent in the hand-inversion agitation technique employed in the routine method.

TABLE IV.—SUMMARY OF METHOD VARIATION IN DISSOLUTION RATE IN SECONDS

	Routine Method	Machine Method
Average of all determinations	42.5	40.7
Maximum deviations from operator to operator	49.1	2.9
Average deviation from operator to operator	20.5	1.4

CONCLUSIONS

1. An apparatus for determining dissolution rates of soluble tablets is described.

2. The importance of control of agitation in dissolution rate determination is shown. This factor can be closely regulated by the instrument described and dissolution rate results achieved by its use are more accurate and reproducible than those obtained when operator judgment governs inversion rate.

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The Estimation of Betaine and Choline in Mixtures*

By F. J. BANDELIN and R. E. PANKRATZ

Choline and betaine may be separated from mixtures of these two lipotropic agents by selective precipitation of their respective reineckates. Choline reineckate may be quantitatively precipitated from an alkaline solution without interference of betaine. Betaine reineckate may be quantitatively precipitated from the acidified solution. Methionine and inositol do not interfere. Accompanying data indicate the method is rapid, accurate, and reproducible.

BETAINE, like choline, has been known for many years as a common constituent of plant and animal tissues. The nutritional significance of these quaternary ammonium compounds was first brought to light when duVigneaud and his co-workers demonstrated the importance of compounds, containing physiologically labile methyl groups. The importance of these methyl donor compounds in preventing fatty deposits in the liver and hemorrhagic degeneration of the kidney has since been pointed out by many investigators.

While choline is apparently utilized in the biological synthesis of phospholipids, betaine appears to be important as an intermediate in the transport of methyl groups in transmethylation. These and other important physiological and nutritional ramifications of these compounds coupled with their availability in pure, synthetic form, make their combined use in pharmaceutical products a distinct probability.

Because of their related chemical structure, choline and betaine react similarly; hence their separation and individual determination in mixtures presents a problem.

In previous work it was demonstrated that choline could be determined in the presence of various vitamins of the B-complex by precipitating it as the reineckate from alkaline solutions (1). Preliminary investigation indicated that betaine forms a reineckate insoluble in acid solution but soluble in alkaline medium. Using this as a basis for the separation of these two compounds we have developed the following procedure.

EXPERIMENTAL

Reagents.—*Choline chloride–betaine hydrochloride solution.*—Dissolve 1.0 Gram of choline chloride (U. S. P. reference standard, stored in a desiccator) and 1.0 Gram of betaine hydrochloride (Eastman Kodak Co.) in distilled water in a 1 L. volumetric flask and adjust the volume to exactly 1 L. with water. Each ml. of this solution contains 1.0 mg. of choline chloride and 1.0 mg. of betaine hydrochloride.

Ammonium reineckate reagent.—One Gram of ammonium reineckate is shaken with 50 ml. of distilled water, then filtered.

Hydrochloric acid, 3 N.

Trisodium phosphate, 25% solution.

Dilute reineckate–trisodium phosphate solution.—Two ml. of saturated ammonium reineckate solution in 1 L. of 5% trisodium phosphate solution.

Acetone, U. S. P.

Water, 25%, in acetone.

PROCEDURE FOR CHOLINE

Into a 15-ml. conical centrifuge tube pipette a solution containing both choline and betaine in concentrations of from 3 to 8 mg. in 5 ml. of solution. Add 1 ml. of 25% trisodium phosphate solution and 5 ml. of ammonium reineckate reagent. Set aside in an ice bath for one hour to assure complete precipitation, and then centrifuge for ten to twenty minutes. Carefully decant the clear solution and wash the precipitate with 2 ml. of dilute reineckate–trisodium phosphate solution. Blow the wash solution from the pipette to break up the packed precipitate. Centrifuge again and decant the clear solution, adding it to the solution first decanted from the precipitate. Dissolve the precipitate of choline reineckate in acetone and adjust the volume to exactly 10 ml. with acetone. Determine the optical density of the solution at 525 mμ in a spectrophotometer and calculate the amount of choline from a similarly treated standard containing exactly 5 mg. of choline chloride from the following expression:

$$0.005 \times \frac{\text{O. D. of choline sample}}{\text{O. D. of choline standard}} = \text{Grams of choline chloride in aliquot}$$

PROCEDURE FOR BETAINE

To the decanted solution and washing from the choline determination in a 15-ml. centrifuge tube, add 1 ml. of dilute hydrochloric acid and set aside in an ice bath for one hour to assure complete precipitation. Centrifuge for twenty minutes and decant the clear supernatant liquid. Invert the tube and allow to stand in the inverted position on an absorbent paper towel to drain as completely as possible. Dissolve the precipitate of betaine reineckate in acetone–water mixture and adjust

* Received January 19, 1953, from the Control Laboratories of Flint, Eaton and Co., Decatur, Ill.

the volume to exactly 10 ml. Determine the optical density of the solution at exactly 525 mμ in a spectrophotometer and calculate the amount of betaine from a similarly treated standard containing exactly 5 mg. of betaine from the following expression:

$$0.005 \times \frac{\text{O. D. betaine sample}}{\text{O. D. betaine standard}} = \text{Grams of betaine in aliquot}$$

Results of a number of determinations by the above procedure on a solution containing equal quantities of choline chloride and betaine hydrochloride (1.0 mg. each per ml.) are given in Table I.

TABLE I.—RECOVERY OF BETAINE AND CHOLINE IN MIXTURES AS REINECKATES

Choline Chloride			Betaine Hydrochloride		
O. D. Sample	O. D. Std.	Per Cent Recovered	O. D. Sample	O. D. Std.	Per Cent Recovered
0.380	0.382	99.4	0.446	0.443	100.6
0.380	0.382	99.4	0.448	0.444	100.9
0.378	0.380	99.4	0.444	0.443	100.2
0.376	0.378	99.4	0.442	0.442	100.0
0.377	0.378	99.7	0.443	0.443	100.0
0.376	0.376	100.0	0.442	0.441	100.2
0.376	0.376	100.0	0.441	0.438	100.6
0.385	0.386	99.7	0.450	0.445	101.1
0.380	0.386	98.4	0.449	0.445	100.9
0.376	0.378	99.4	0.443	0.442	100.2

DISCUSSION

The solubility of betaine reineckate in alkaline solution is undoubtedly due to the presence of the carboxyl group in betaine which, in alkaline solution, confers solubility upon the reineckate through the formation of the sodium salt. Washing of the betaine reineckate without some slight loss was not found possible. For this reason it is taken up in the acetone–water mixture, after centrifuging, without washing. Attempts to set up a standard curve for betaine reineckate were not completely successful because of this solubility. Repeated attempts at establishing a standard curve using increasing amounts of betaine indicated that variations in the slope of the curve could not be overcome altogether. Betaine reineckate does, however, follow Beer's law, within range of the variation, with the line always intersecting the X and Y axis origin. For this reason, calculations for the determination have been based upon the operations simultaneously with the sample and in an identical manner. The use of an internal standard where a known quantity of betaine is added to a second aliquot of the unknown is also recommended.

Choline results in the determination are reproducible and these determinations may be based upon or calculated from a standard curve.

Data given in Table I indicate that the method is reliable and reproducible. Accuracy within 1% may be expected.

Methionine and inositol, often used in combinations of lipotropic compounds do not interfere. Certain vitamins of the B complex such as pyridoxine and nicotinic acid, however, do interfere with the determination of betaine. This is usually not a major factor because of the disproportion of the usual dosages of betaine and the vitamins involved.

SUMMARY

1. It is possible to estimate betaine and choline in mixtures by selective precipitation of their reineckates.

2. Choline can be precipitated from alkaline solution without interference of betaine which does not form an insoluble reineckate in the presence of alkali.

3. Betaine can be precipitated by acidifying the solution after removal of the choline reineckate.

4. The method is rapid, accurate and, as indicated by accompanying data, reproducible.

REFERENCE

(1) Bandelin, F. J., and Tuschhoff, J. V., THIS JOURNAL, 40, 245(1951).

Notes

A Note on the Effect of Cortisone and Desoxycorticosterone on Metrazol Convulsions in Mice*,†

By CHARLES A. LEONARD, CHARLES K. GORBY, JULIAN L. AMBRUS, and JOSEPH W. E. HARRISSON

THE WIDESPREAD interest in the steroid hormones has led to experiments involving their use in conditions other than specific hormonal deficiencies. The anticonvulsant activity of desoxycorticosterone in animals has been reported by several authors (1, 2) using electric stimulation methods to produce the convulsions (3, 4). Aird (5) produced convulsions in mice with cocaine hydrochloride, and in cats by means of electric stimulation. Injection of desoxycorticosterone (DCA) in a dosage of 6 mg./Kg. i. p. for three days prior to administration of cocaine (injected twenty-four hours after the last dose of DCA) resulted in virtually no anticonvulsant effect. However, when the same dosage of desoxycorticosterone was injected thirty minutes prior to the cocaine, a definite anticonvulsant action was manifested. Convulsions produced by electric stimulation of cats were not affected by a four-day pretreatment with desoxycorticosterone at a dosage of 1 mg. per day.

Lowell, *et al.* (6), reported the occurrence of convulsive seizures in humans during the treatment of asthma with cortisone acetate, and other observers have noted convulsive seizures in patients receiving ACTH. Prolonged administration of ACTH to rats induced a decrease in sensitivity to Metrazol, according to Torda and Wolff (7).

The antagonism of adrenal-cortical extracts and cortisone to desoxycorticosterone in adrenalectomized-DCA-implanted rats was demonstrated by Woodbury, *et al.* (8), by measuring the electroshock threshold (3, 4). These workers suggest that the nature of this antagonism may be a form of competitive antagonism due to similarity in chemical structure. The effect of adrenocortical steroids and ACTH on electroshock seizure thresholds in rats when administered alone and in combination has been recently reported by Woodbury (9).

In view of the above, an experiment was undertaken to determine the effect of cortisone[1] and desoxycorticosterone[2] pretreatment upon Metrazol-induced convulsions in mice. Male Swiss mice (Webster strain) in the weight range of 22 ± 2 Gm. were selected and divided into groups as follows:

Group	Number of Animals	Pretreatment
I	40	None—control
II	40	Cortisone Acetate 10 mg./Kg. s. c. daily for 7 days
III	40	Desoxycorticosterone Acetate 10 mg./Kg. s. c. daily for 7 days

TABLE I.—EFFECT OF CORTISONE AND DESOXYCORTICOSTERONE ON METRAZOL CONVULSIONS IN MICE

Group	Convulsions	Doubtful	None	Severity
I—Control				
A—Metrazol 25 mg./Kg. i. p.	0	0	10	...
B—Metrazol 35 mg./Kg. i. p.	2	0	8	Slight
C—Metrazol 45 mg./Kg. i. p.	6	1	3	Marked
D—Metrazol 55 mg./Kg. i. p.	10	0	0	Marked
II—Cortisone Pretreated				
A—Metrazol 25 mg./Kg. i. p.	0	0	10	...
B—Metrazol 35 mg./Kg. i. p.	3	1	6	Slight
C—Metrazol 45 mg./Kg. i. p.	7	0	3	Marked
D—Metrazol 55 mg./Kg. i. p.	9	1	0	Marked
III—DCA Pretreated				
A—Metrazol 25 mg./Kg. i. p.	0	0	10	...
B—Metrazol 35 mg./Kg. i. p.	0	0	10	...
C—Metrazol 45 mg./Kg. i. p.	8	0	2	Marked
D—Metrazol 55 mg./Kg. i. p.	9	1	0	Marked

* Received September 10, 1952, from the LaWall Memorial Laboratory of Biochemistry and Pharmacology, Philadelphia, Pa.

† This study was supported in part by a research grant from Eli Lilly and Company, Indianapolis, Ind.

[1] Cortisone (Cortone Acetate) was generously supplied by Merck and Co., Rahway, N. J.

[2] Desoxycorticosterone (Desoxycorticosterone Acetate) was generously supplied by Schering Corporation, Bloomfield, N. J.

The dosages of cortisone and desoxycorticosterone selected for this experiment are higher than those used by Aird (5). The dosages administered to the mice in this experiment correspond on a weight comparison basis to 700 mg. for a 70 Kg. man.

Following pretreatment, each group was subdivided into four groups of ten animals. Each subgroup received the following doses of Metrazol:

A—Metrazol 25 mg./Kg. i. p.
B—Metrazol 35 mg./Kg. i. p.
C—Metrazol 45 mg./Kg. i. p.
D—Metrazol 55 mg./Kg. i. p.

These dosages ranged from the CD_0 to the CD_{100} (Convulsive Dose) as previously determined for this strain of mice in our laboratory. Metrazol injections were made four hours after the last pretreatment dose of cortisone and desoxycorticosterone. Following the injection of Metrazol, observations were made upon the time of onset, severity, and duration of the convulsion. Mortality was not manifested in the dosage used in this experiment. The data are given in Table I.

SUMMARY

From the observations made during this experiment it appears that pretreatment with cortisone and desoxycorticosterone does not affect Metrazol-induced convulsions in mice to a significant degree.

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(4) Stone, W. E., Webster, J. E., and Gurdjian, E. S., *J. Neurophysiol.*, **9,** 231(1946).
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(8) Woodbury, D. M., *et al.*, *Proc. Soc. Exp. Biol. Med.*, **76,** 65(1951).
(9) Woodbury, D. M., *J. Pharmacol. Exptl. Therap.*, **105,** 27(1952).

A Note on a Contraindication to Propylene Glycol, as a Solvent in Toxicity Studies*

By KENNETH F. LAMPE and OTHO D. EASTERDAY

THE LITERATURE indicates that propylene glycol is relatively free of toxicity. It was found, however, during a recent series of toxicity studies in this laboratory that the propylene glycol used as a vehicle for the drug was producing convulsions on intraperitoneal injection in mice. The question of a species difference raised by Morris, *et al.* (1), appears to be indicated since only sedation is produced in the rat.

The administration of a toxic intraperitoneal dose of propylene glycol in albino white mice produced an immediate irritation at the sight of injection, followed by hypersensitivity to noise and touch. The reported observation of loss of equilibrium (2) was noted in this experiment. A period of deep narcosis, followed by violent, short, unsymmetrical convulsions preceded death. These observations confirm those described by other workers (2–4). An attempt to evaluate the level of convulsive activity by selective dissection of the frog following administration of propylene glycol into the ventral lymph sac seemed to indicate portions on the central nervous system other than the cord.

The LD_{50} for propylene glycol in mice by intraperitoneal injection was found by the method of probits to be 9.33 ml./Kg. or 9.70 Gm./Kg. This LD_{50} dose confirms that reported by Karel, *et al.* (5), who found a median lethal dose of 9.36 ml./Kg. or or 9.73 Gm./Kg.

Although it is apparently justifiable to use propylene glycol as a solvent for oral administration in humans without regard to toxicity, the central activity of this compound warrants more thorough investigation. Propylene glycol appears *not* to be a suitable choice as a solvent in toxicity studies, particularly in mice.

* Received December 23, 1952, from the Department of Pharmacology, College of Medicine, The State University of Iowa, Iowa City, Iowa.

REFERENCES

(1) Morris, H. J., Nelson, A. A., and Calvery, H. O., *J. Pharmacol. Exptl. Therap.*, **74,** 266(1942).
(2) Braun, H. A., and Cartland, C. F., THIS JOURNAL, **25,** 746(1936).
(3) Hanzlik, P. J., Newman, H. W., Van Winkle, W., Lehman, A. J., and Kennedy, N. K., *J. Pharmacol. Exptl. Therap.*, **67,** 101(1939).
(4) Seidenfeld, M. A., and Hanzlik, P. J., *ibid.*, **44,** 109 (1952).
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United States Pharmacopoeial Convention

THIRD ANNUAL FINANCIAL STATEMENT BASED UPON THE REPORT OF THE AUDITOR FOR THE YEAR ENDED DECEMBER 31, 1952

STATEMENT OF INCOME AND EXPENSE (GENERAL FUND)

Income			
Sale of Pharmacopoeias:			
Collections		$61,647.00	
Add: Increase in Accounts Receivable:			
December 31, 1952	$ 6,980.00		
December 31, 1951	6,948.80	31.20	$61,678.20
Sale of Reference Standards A. M. A. Articles, etc.:			
Collections		$15,669.63	
Add: Increase in Accounts Receivable:			
December 31, 1952	$ 1,577.25		
December 31, 1951	1,510.85	66.40	
		$15,736.03	
Less: Adjustment of Prior Year's Collections		2.73	15,733.30
Interest on Investments:		$ 6,862.50	
Less: Amortization of Bond Premium		37.18	6,825.32
Use of Text by Others			50.00
Miscellaneous			93.50
Total Income			$84,380.32
Less—Expense:			
Revision		$52,979.49	
Add: Decrease in Inventory:			
December 31, 1951	$16,240.09		
December 31, 1952	15,475.29	764.80	$53,744.29
Administration			6,977.19
Publication and Sales Expense		$ 1,492.00	
Add: Decrease in Inventory:			
December 31, 1951	$26,849.92		
December 31, 1952	3,039.47	23,810.45	25,302.45
Convention			400.00
Headquarters—New York		$ 6,095.86	
Add: New York Office Depreciation Expense:			
Building	$ 2,430.77		
Furniture and Equipment	2,670.45	5,101.22	11,197.08
Total Expense			97,621.01
Excess of Expenses over Income			$13,240.69

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNTS)

Cash on Deposit, December 31, 1951		$190,164.33
Add—Receipts:		
Sales of Pharmacopoeias	$61,647.00	
Sales of Reference Standards, A. M. A. Articles, etc.	15,669.63	
Interest on Investments	6,862.50	
Use of Text by Others	50.00	
Miscellaneous	93.50	84,322.63
		$274,486.96

Less—Disbursements:

	Revision	Administration	Publication and Sales	Convention	Headquarters	Total
Printing and Binding	$	$	$1,482.00	$	$	$ 1,482.00
Salaries	31,945.10	3,984.00	.....	400.00	1,320.00	37,649.10
Meetings	4,764.89	601.94	.....	.....	.....	5,366.83
Supplies	1,786.34	62.36	.....	.....	.....	1,848.70
Postage and Telegrams	1,203.75	39.89	.....	.....	.....	1,243.64
Utilities	.....	.....	.....	.....	2,075.43	2,075.43

(Continued on next page)

	Revision	Administration	Publication and Sales	Convention	Headquarters	Total	
Repairs and maintenance	.....	.....	.....	.....	2,199.28	2,199.28	
General	13,279.41[a]	2,289.00[b]	10.00	.....	.....	15,578.41	
Insurance	.....	.....	.....	.....	501.15	501.15	
Furnishings & Equipment	.....	.....	.....	.....	88.53	88.53	
	$52,979.49	$6,977.19	$1,492.00	$400.00	$6,184.39		68,033.07
Cash on Deposit, December 31, 1952							$206,453.89

[a] Retirement & Social Security	$ 6,501.62	[b] Auditor	$ 450.00
Reference Standards Stock	2,797.18	Counsel	600.00
Assistant, Supplies, etc. Dr. Beal's Subcommittee	3,208.33	Kelly Memorial Conference Room	1,000.00
Miscellaneous	772.28	Miscellaneous	239.00
	$13,279.41		$2,289.00

Book Notices

Commercial Directory of Africa, 1950–1951. International Marketing Service Corp., Nairobi, Kenya Colony, Africa, 1951. xxiv + 279 pp. 24.5 x 16 cm. Price $5.

This *Directory* should be of value to all commercial and industrial concerns having business relations with the various territories and countries of Africa. It might also serve certain business enterprises in the United States which may be exploring fresh fields for development and new connections.

Pharmaceutical Formulas. 12th ed., Vol. I. The Chemist and Druggist, 28 Essex St., Strand, London, W.C. 2, England, 1953. xv + 1139 pp. 14 x 21 cm. Illustrated. Price 37*s.* 6*d.*

The first edition of *Pharmaceutical Formulas* was published in 1898. It has undergone eleven revisions, and the twelfth edition has been revised completely with the incorporation of much new material. The book consists of a collection of several thousands of formulas which pharmacists and others in the pharmaceutical profession may require at one time or another. The formulas cover the entire field of galenical preparations and include material selected from 26 national pharmacopeias and many formularies including *The National Formulary.* Quite a large proportion of the formulas have been obtained from medical and pharmaceutical publications and other comparable sources.

In the course of the revision, many of the older formulas have been omitted if they appear to be obsolete. A useful feature of the book is the inclusion of formulas for preparations or prescriptions designated in the literature by the name of the author or originator. The book also includes a table of equivalent names of official titles and synonyms used in different pharmacopeias. Another table gives the concentrations of acids, alcohols, and alcohol solutions required in the pharmacopeias and other official drug compendia such as the National Formulary which occupies an official position equal to that of the U. S. P.

While the book is designed primarily for use by British pharmacists, it is equally valuable as a book of reference for members of the pharmaceutical profession in all the countries of North and South America.

British Pharmacopoeia 1953. Published under the direction of the General Medical Council. The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C. 1, 1953. xxiv + 894 pp. 15.5 x 23.5 cm. Price 50*s.*

The *British Pharmacopoeia* was first published in 1864 and the 1953 edition is the eighth. It is also the first edition to appear since the five-year revision period was adopted.

As a result of the short period between the seventh and eighth editions, only 63 new monographs were added since 1948, although 158 monographs were deleted.

The most notable difference in the *British Pharmacopoeia 1953* is the use of English in the main titles of the monographs. Familiarity with titles in this language should enable the development of a more logical arrangement of the text in the next revision. Monographs on basic drugs, as distinct from preparations, are arranged alphabetically. Under the Gen-

eral Notices is the statement: "Titles which may be derived by the suitable inversion of words of a main or subsidiary English title are also official titles. Thus, Tablets of Aspirin, Aspirin Tablets, Ointment of Zinc Oxide, and Zinc Oxide Ointment are all official titles." Having indicated an awareness that dosage forms can be and are referred to with the name of the medicinal ingredient first, and having the texts of the U. S. P. and N. F. as guides, the British Pharmacopoeia Commission proceeded to evolve a confusing arrangement of monographs of dosage forms. The monograph on Cascara Sagrada is followed by the one for the Dry Extract of Cascara, which is followed by Tablets of Cascara Sagrada. The reason for Tablets following the Dry Extract is that the Dry Extract is used in the preparation of the Tablets. The Tablets monograph is then followed by those for the Elixir and for the Liquid Extract of Cascara Sagrada. The monograph for Sodium Potassium Tartrate is followed by Compound Effervescent Powder. A marginal listing could be very helpful with such an arrangement and none is given. The situation is saved by a thorough Index in which all entries are alphabetically arranged and cross-indexed.

The fact that no Latin subtitles were used in the monographs that have been included since the 1951 *Addendum to British Pharmacopoeia 1948*, might be an indication that all Latin forms will eventually be eliminated. The peculiar Latinized forms that are used in the English language compendia will not be missed by most practitioners in the medical care professions.

Two dosage forms appear in the *British Pharmacopoeia* for the first time, namely Capsules and Implants. "Application of Benzyl Benzoate" utilizes 2 per cent of Emulsifying Wax to prepare the counterpart of the U. S. P. Benzyl Benzoate Lotion.

The British, too, are still trying to find the most suitable formula for Calamine Lotion. The latest effort utilizes, as the suspending agent, 3 per cent of Bentonite, added as a powder, and includes 0.5 per cent of Liquefied Phenol.

In all its technical aspects, the *British Pharmacopoeia 1953* is an excellent book of standards. It does not differ much from the 1948 edition, although the arsenic limits are now determined as arsenic and not as arsenic trioxide. A new section on statistical design for biological assays is included.

The members of the British Pharmacopoeia Commission and those who assisted in the compilation of the *British Pharmacopoeia 1953* have well performed their task.

Substances Naturelles de Synthèse. Vol. V. By LÉON VELLUZ. Masson et Cie, Paris VI^e, 1953. 16 x 23.5 cm. 206 pp. Illustrated. Price 1,500 fr.

The fifth volume in this series of French publications presents detailed methods of synthesis for calciferol, papaverine, DL-phenylalanine, phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone), α-phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone), and phytol. Methods for 7-dehydrocholesterol and homoveratronitrile are discussed.

Compounds containing quinoline and isoquinoline rings are examined as are addition reactions with diene-containing molecules. The survey of chromatographic adsorption that appeared in the fourth volume is followed by a discussion of partition chromatography in this volume.

The other volumes of this series were reviewed in THIS JOURNAL, **40,** 534(1951); **41,** 167, 630(1952). This volume contains a composite index for the five volumes that have been published.

Les Détersifs en Pharmacie. By W. KOPACZEWSKI. Masson et Cie., Paris, 1953. 170 pp. 16 x 25 cm.

This book, in French, is a discussion of surface-active substances with particular emphasis on their relation to colloidal and biological systems. The text is divided into three parts with the following headings and chapter titles: *A.* Surface active substances and conditions of colloidal equilibrium. Natural organic substances (saponins, etc.); synthetic substances (alkyl aryl sulfonates, nonionic compounds, etc.); relation of degree of dispersion and colloidal equilibrium; degree of hydration in colloidal gels. *B.* Permeability. Physicochemical aspect (composition of protoplasm and role of physical factors); biological resorption (permeability of various tissues). *C.* Applications. Gels, creams, and ointments. Antiseptic power of surface-active agents. Tables of physical constants are appended.

The table of contents appears at the end of the book. A bibliography is given at the end of each chapter, but there is no index to the text.

BOOKS RECEIVED

The Bacteriostatic Activity of 3500 Organic Compounds for Mycobacterium Tuberculosis Var. Hominis. By GUY P. YOUMANS, LEONARD DAUB, and ANNE S. YOUMANS. Chemical-Biological Coordination Center, National Research Council, Washington, D. C., 1953. v + 713 pp. 17.5 x 25 cm. Price $5.

Basic Drugs. U. S. Public Health Service, Hospitals and Clinics, 1953. Federal Security Agency, Public Health Service. Public Health Service Publication No. 246. Superintendent of Documents, U. S. Government Printing Office, Washington 25, D. C. iv + 165 pp. 15 x 24 cm. Price $0.50.

Physiological and Therapeutic Effects of Corticotropin (ACTH) and Cortisone. By DWIGHT J. INGLE and BURTON L. BAKER. Publication No. 179 of "American Lectures in Metabolism." Published by Charles C Thomas, Springfield, Ill., 1953. xii + 172 pp. 14.5 x 22.5 cm. Price $5.50.

Medical Public Relations, A Study of the Public Relations Program of the Academy of Medicine of Toledo and Lucas County, Ohio, 1951. By EDGAR A. SCHULER, ROBERT J. MOWITZ, and ALBERT J. MAYER. Edwards Brothers, Inc., Ann Arbor, Mich., 1952. xiv + 228 pp. 16 x 23.5 cm.

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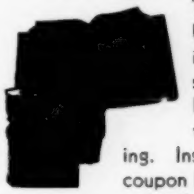

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